Extended spectrum beta-lactamase producing Enterobacteriaceae – aspects on carriage, infection and treatment

Thesis by

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Arne Søraas

List of papers

1 **Risk factors for community-acquired urinary tract infections caused by ESBL-producing Enterobacteriaceae – a case–control study in a low prevalence country**
Arne Søraas, Arnfinn Sundsfjord, Irene Sandven, Cathrine Brunborg, Pål A. Jenum
PLOS ONE 10.1371/journal.pone.0069581

2 **Extended spectrum betalactamase-producing bacteria are not detected in oral samples from human fecal carriers of ESBL-producing Enterobacteriaceae**
Arne Søraas, Ingar Olsen, Arnfinn Sundsfjord, Trude Handal, Ola Bjørang, Pål A. Jenum
Submitted

3 **High rate of per oral mecillinam treatment failure in community-acquired urinary tract infections caused by ESBL-producing *Escherichia coli***
Arne Søraas, Arnfinn Sundsfjord, Silje Bakken Jørgensen, Knut Liestøl, Pål A. Jenum
PLOS ONE 10.1371/journal.pone.0085889

Abbreviations

bla	Gene encoding beta-lactamase
BSI	Blood stream infections
CA-UTI	Community acquired urinary tract infection
CCUG	Culture Collection, University of Goteborg
CI	Confidence interval
CTX-M	Cefotaximase Munich (a group of beta-lactamases)
DDD	Defined daily dose
ESBL	Extended spectrum beta-lactamase
$ESBL_A$	Extended spectrum beta-lactamase – "classic" Ambler class A
$ESBL_{CARBA}$	Extended spectrum beta-lactamase – carbapenemase
ESBL-E	Extended spectrum beta-lactamase producing Enterobacteriaceae
$ESBL_M$	Extended spectrum beta-lactamase - miscellaneous
ESCAPE	Extended Spectrum beta-lactamases – Carriage, environmental dissemination And Population Epidemiology (abbreviation used for the project that the PhD is a part of)
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
KPC	*Klebsiella pneumoniae* carbapenemase
MIC	Mean inhibitory concentration
NDM	New Delhi metallo-beta-lactamase
OR	Odds ratio
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
RHAN	Rhanella
ROC curve	Receiver operating characteristic curve
SHV	Sulfhydryl variable (a group of beta-lactamases)
TE-buffer	Tris-EDTA buffer
TEM	Temoneira (a group of beta-lactamases named after a patient)
UTI	Urinary tract infection

Introduction

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."

- Alexander Fleming in his Nobel Lecture "Penicillin", December 11, 1945

Bacterial antimicrobial resistance

The introduction of antibiotics around World War II represented a revolution in therapeutic medicine and has saved millions of lives. When penicillin was introduced in the 1940s a "golden age" of antibiotic discovery began and during the next three decades several new classes of antimicrobial agents with different targets of action were introduced to the market[1]:

Milestones in the introduction to the market of new antibiotic classes[1,2]

1935 – sulfonamides
1941 – beta-lactams
1944 – aminoglycosides
1950 – tetracyclines
1952 – macrolides/lincosamides/streptogramins
1956 – glycopeptides
1959 – nitroimidazoles
1962 – quinolones
1968 – dihydrofolate reductase inhibitors
2000 – oxazolidinone
2003 – lipopeptides

Milestones in the history of beta-lactams

1941 – penicillin (the first beta-lactam)
1961 – ampicillin (a broad spectrum penicillin)
1962 – cephalosporins (a new group of beta-lactams)
1985 – carbapenems (a new group of beta-lactams)

With the exceptions of the oxazolidinones and lipopeptides, drugs with a gram-positive antimicrobial spectrum, new antibiotics introduced since 1968 have been chemical

modifications of existing drug scaffolds and no new classes of gram-negative drugs have been introduced. This is partly a result of diminishing research into new antibiotics. There are several reasons for this including: i) the 1962 "Drug Efficacy Amendment" to the Federal Food, Drug, and Cosmetic Act, in the USA which set stricter rules on new drugs, ii) rules that new drugs should be non-inferior to existing ones, iii) the use of new antibiotics as “last-resort” drugs only and iv) the fact that antibiotics are mostly used in short courses (i.e., a lower quantity of drugs are sold compared to lifelong treatment of other drug groups). In parallel with the reduced pipeline of new drugs the use of antibiotics among humans and animals have risen dramatically. Predictably, this has led to an ever increasing problem of antimicrobial resistance in bacteria[3,4].

The term antimicrobial resistance most often refers to resistance that is acquired by bacteria, as opposed to intrinsic resistance in which some antimicrobials do not affect the large majority of strains from certain bacterial species.

Whether a strain of bacteria is resistant to an antibiotic or not is determined by its mean inhibitory concentration (MIC) value. This value is measured under standardised conditions and is usually based on observed growth of the strain in a broth or on an agar plate containing antibiotics (Figure 1).

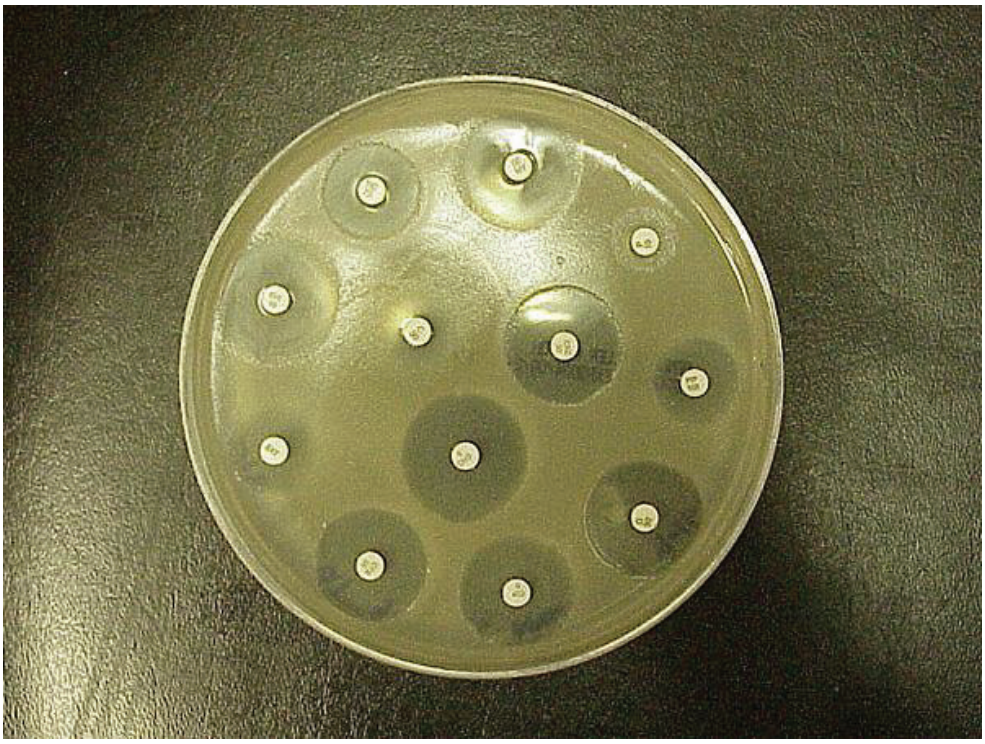


Figure 1.
Agar plate with antibiotic discs and clear zones without bacterial growth around the discs. Bacterial resistance may be determined based on zone diameter under standardized conditions. (Picture: Public domain, Wikimedia, thanks to oceanexplorer.noaa.gov)

If a strain has a MIC above a certain level, it is defined as resistant to that antibiotic. The value of the cut-off point is set by national or international bodies like The European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) (www.eucast.org and www.clsi.org) and is called the clinical breakpoint. Predetermined procedures are used when determining these breakpoints and they are based on whether a level of antimicrobial activity is associated with a high likelihood of therapeutic success or failure in patient treatment[5]. In certain cases epidemiological cut-off values (ECOFFs) are also of interest. These are the upper value of the "wild type population" MIC distribution (i.e., the strains without any acquired resistance mechanisms). Examples of situations when the ECOFF value is of interest are in research and when no clinical breakpoints have been defined.

The increase of antibiotic resistance is a serious problem in medicine and may threaten to end the so called antibiotic era[6]. This has been the topic of recent reports from important stakeholders like the European Centre for Disease Prevention and Control, the American governmental organization Centre of Disease Control and Prevention and the Swiss independent forum World Economic Forum[7-10].

This thesis focuses on extended spectrum beta-lactamase (ESBL), a form of acquired resistance to most beta-lactams – our most valuable group of antibiotics. This affects common pathogenic gram-negative bacteria like *Escherichia coli* or *Klebsiella pneumoniae*. The aims of the papers presented include i) exploring risk factors for community acquired urinary tract infection (CA-UTI) caused by these bacteria, ii) investigating of oral carriage of bacteria with ESBL and iii) evaluating the outcome of mecillinam treatment against CA-UTI caused by these bacteria.

Mechanisms of antimicrobial resistance

Genetic basis of resistance

The basis for all resistance is the genetic makeup of the bacteria. A bacterium may acquire resistance through modification of existing genes, changes in gene expression or acquisition of new genes through the process called "horizontal gene transfer". There are three mechanisms of horizontal gene transfer – transduction, transformation and conjugation. Transduction is DNA transfer mediated by bacteriophages and transformation is direct uptake of genetic material, including plasmids from the environment by "competent" bacteria. This thesis

mainly addresses *conjugation* in which two bacteria connect and genetic information - usually in the form of a plasmid - is transferred from one to another (Figure 2). Plasmids are extrachromosomal DNA molecules that may be found in many bacterial, archaea and eukaryotic species. Plasmids are separate from, and can replicate independently of, the host cells´ chromosomal DNA. They often code for genes thought to be favourable to the host, e.g., antimicrobial resistance genes for genes enabling conjugation. Previously, plasmids carrying antimicrobial resistance genes were known as "resistance-factors" or "R-factors". Plasmids are divided into incompatibility groups ("Inc") based on the type of plasmids that may be harboured in the same bacteria at the same time (two different plasmids from the same Inc group may not be harboured in one bacterium). More than 30 such groups are known, and ESBL genes are often harboured on plasmids belonging to IncF, I or N groups. Plasmids are a key factor behind the success of the ESBL genes as they both facilitate transfer and stable maintenance of these genes in new hosts.

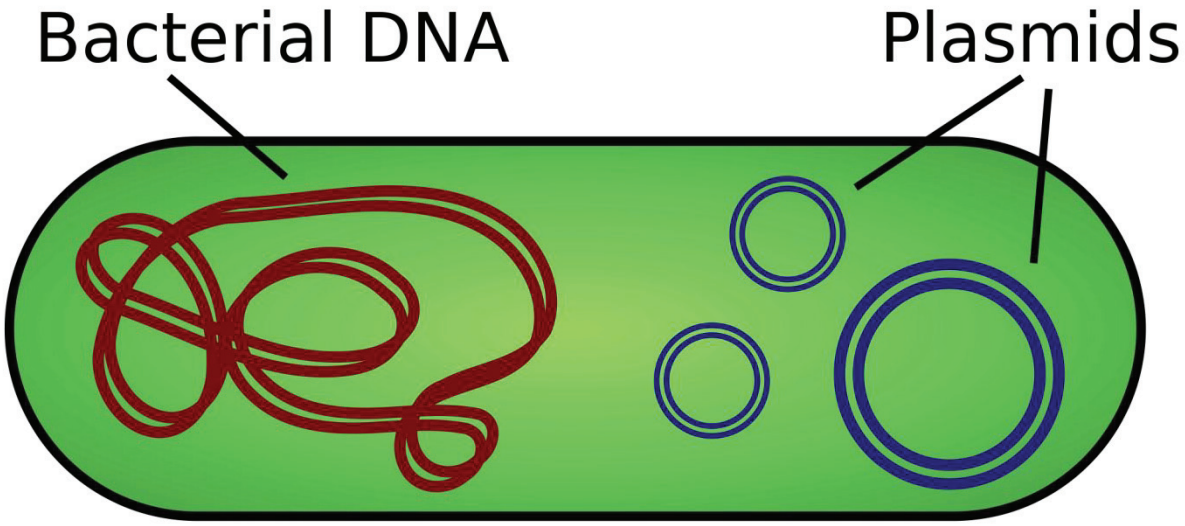


Figure 2.
Plasmids inside bacteria (Wikimedia Commons, Attribution-Share license, thanks to Spaully)

Biochemical basis of resistance

The resistance phenotype of the bacteria depends on its gene content and how this is expressed. The resulting biochemical mechanisms of resistance are often divided into groups[11]:

1. Drug inactivation or modification

    Example: *E. coli* producing beta-lactamase that hydrolyse beta-lactam antibiotics (acquired) or *K. pneumoniae* producing similar enzymes destroying ampicillin (intrinsic).

2. Reduction of drug accumulation or access to the cell

    Example: *E. coli* with altered porins or efflux pumps that reduce the antibiotic concentration of tetracyclines (acquired) or anaerobic bacteria that lack oxidative metabolism to drive uptake of aminoglycosides (intrinsic).

3. Alteration of a metabolic pathway

    Example: Sulfonamide resistant bacteria that have changed their metabolism to utilize folic acid instead of para-aminobenzoic acid (acquired).

4. Alteration of the drug target site

    Example: *Staphylococcus aureus* with a modified binding site to which several beta-lactam antibiotics do not bind (i.e., methicillin resistant *S. aureus* (MRSA) (acquired) or enterococci expressing binding site with low affinity to cephalosporins (intrinsic).

Beta-lactams, beta-lactamases and penicillin-binding proteins

Beta-lactams represent a broad class of antibiotics characterized by a beta-lactam ring in their molecular structure (Figure 3)[12].

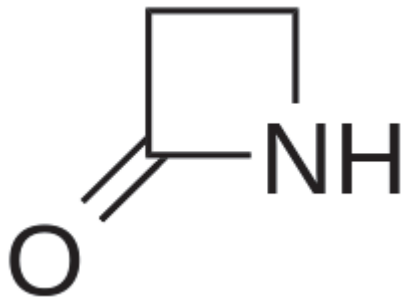


Figure 3.
The beta-lactam ring (2-Azetidinone)

They target the bacterial cell wall synthesis, which has no eukaryotic equivalent. Consequently, they can kill bacteria effectively without exerting a major toxic effect on human cells[13]. They act by binding to "penicillin-binding proteins" (PBPs) in the bacterial cell wall. The PBPs are bacterial enzymes crucial in cell-wall synthesis.

Their main function seems to be crosslinking strands of peptidoglycan into a fishnet-like polymer that shapes the bacteria and withstands their internal osmotic pressure (Figure 4).

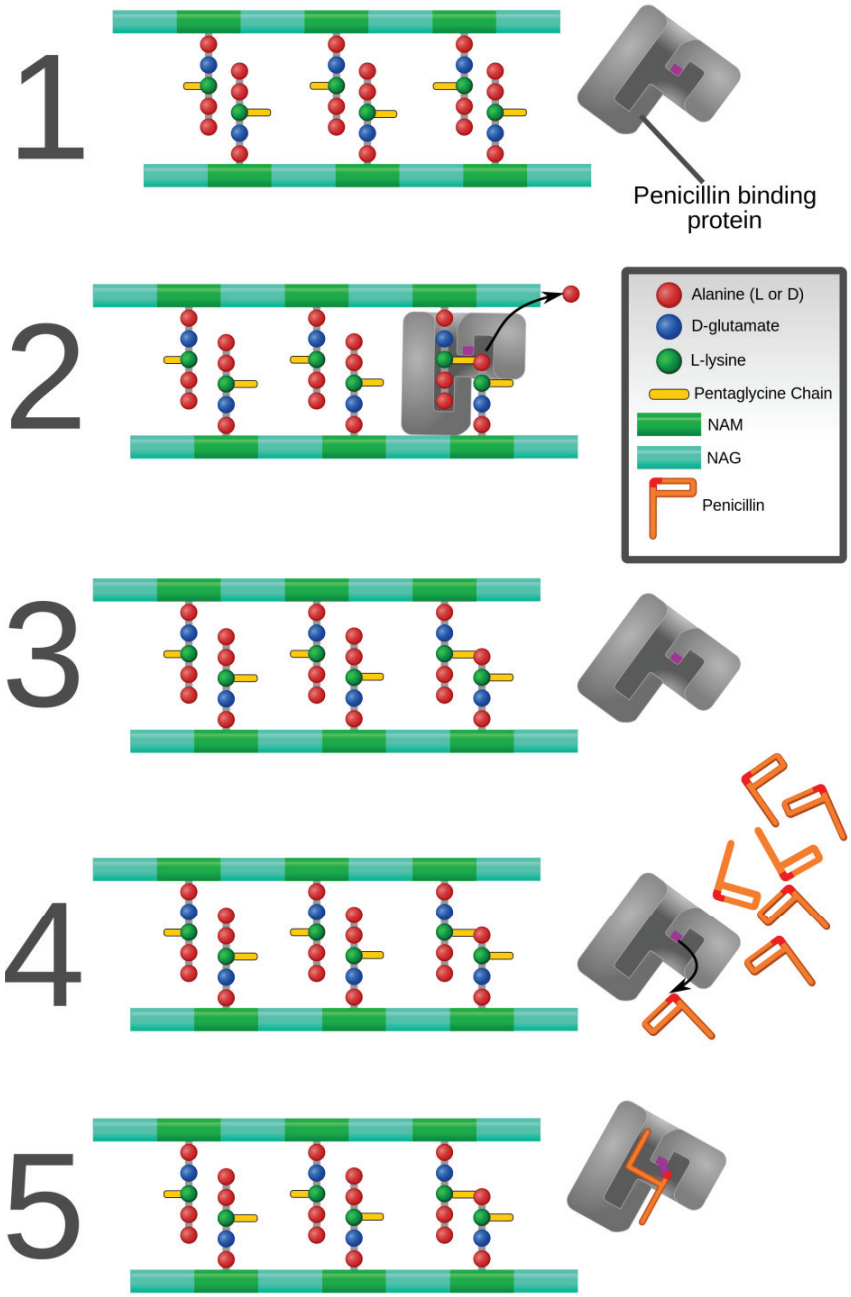


Figure 4.
Penicillin and most other β-lactam antibiotics act by inhibiting penicillin-binding proteins, which normally catalyze cross-linking of bacterial cell walls. (Wikimedia commons, thanks to Mcstrother).

Bacteria usually have several different PBPs (usually numbered PBP 1, 2, 3 and so on according to molecular weight) with partly overlapping functions. Different beta-lactams can act on one or several of these[12]. PBPs also have other and partly unknown vital functions in the bacterial cells, for instance related to division in *Chlamydia trachomatis* reticulate

bodies[14]. When a beta-lactam binds to a PBP, a covalent acyl-enzyme ester bond is formed which only very slowly hydrolyses (deacylates) in the bacteria, and therefore the PBP enzyme is effectively inactivated[12]. The exact mechanisms of how loss of PBP enzymatic activity affects bacteria are poorly understood. Activation of cell wall degenerative enzymes called autolysins are probably involved, leading to activation of lethal cascades for most bacterial species[12,15-17].

Because of their favourable properties, beta-lactams are the most used and also the most important group of antibiotics[18]. In Norway, the use of beta-lactams is stable and in 2012 the beta-lactam group represented 53% of the antibiotics used in humans as measured by the number of defined daily doses (DDD) prescribed[19].

In beta-lactam antibiotics the beta-lactam ring is fused to other molecular ring-structures, and they are classified into subgroups like penicillins, cephalosporins, carbapenems, and monobactams according to the structure of the core ring (Figure 5).

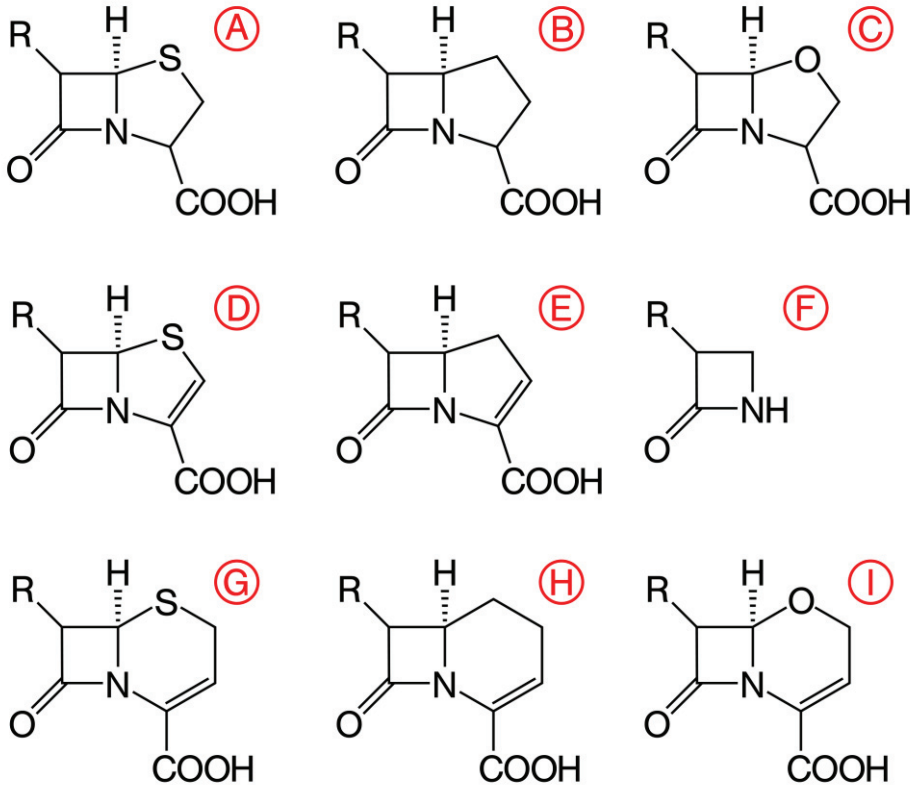


Figure 5.
The β-lactam core structures. (A) A penam. (B) A carbapenam. (C) An oxapenam. (D) A penem. (E) A carbapenem. (F) A monobactam. (G) A cephem. (H) A carbacephem. (I) An oxacephem (Wikimedia Commons, public domain, thanks to Fvasconcellos).

Beta-lactamases

Resistance to beta-lactams is frequently mediated through production of beta-lactamase enzymes which break down beta-lactam molecules. Beta-lactamases are thought to have the same origin as the PBPs, and these enzymes are considered to be one molecular superfamily of proteins that are able to bind beta-lactams. This is exemplified by crystal structure comparison between the most prevalent beta-lactamases ("Ambler Class A") with PBPs showing a high similarity to *E. coli* PBP 4 and 5 and also PBPs from several other bacteria. This superfamily is probably very ancient and may have originated more than 2 billion years ago[20]. The beta-lactamases act by binding to beta-lactams, but the rate of deacylation is up to a million times faster than between a beta-lactam and a PBP and upon deacylation the beta-lactam-ring is opened resulting in inactivation of the beta-lactam molecule[12,21]. A beta-lactam will therefore rapidly be inactivated when attached to an appropriate beta-lactamase.

Beta-lactamases can have different locations in bacteria and may also be excreted. In gram-negative bacteria they are mostly located in the periplasmatic space (Figure 6)[12].

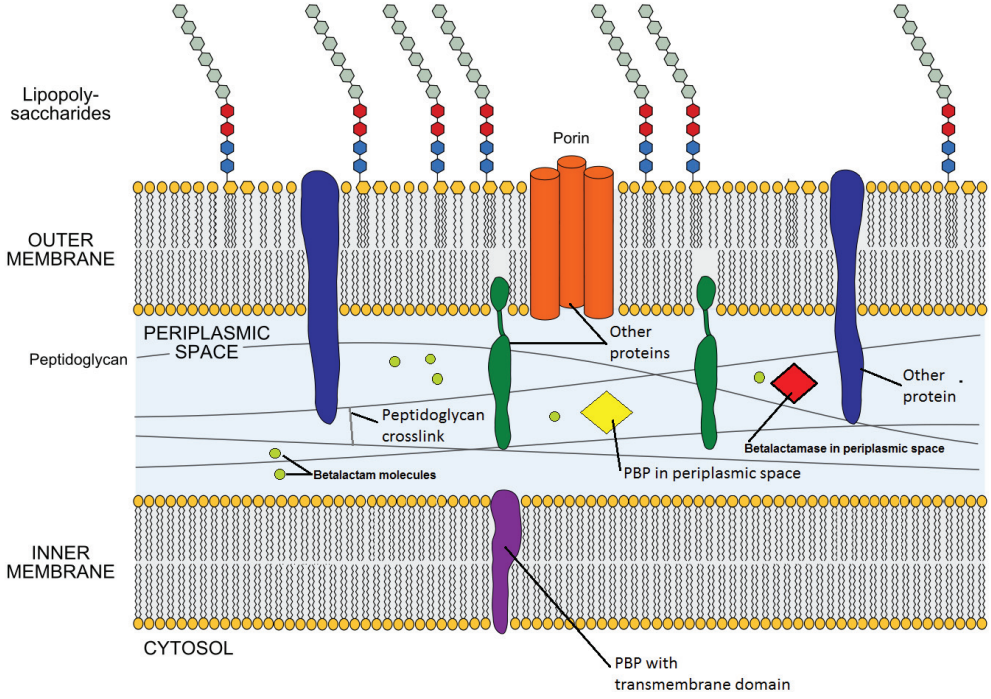


Figure 6.
The gram-negative cell wall with location of two different penicillin-binding proteins, one beta-lactamase and beta-lactams (Wikimedia Commons, modified from Jeff Dahl - thank you)

R.P. Ambler divided the beta-lactamases into four subclasses dependent on their molecular structure: A, B, C and D. Class A, C and D are serine-dependent, i.e., they utilize a transient serine acylation/deacylation at the active site, while class B are dependent on a metal ion at the active site (Figure 7)[22]. Beta-lactamases may also be divided into groups according to their preferred substrates and inhibitors. ESBLs may come from all the Ambler molecular classes.

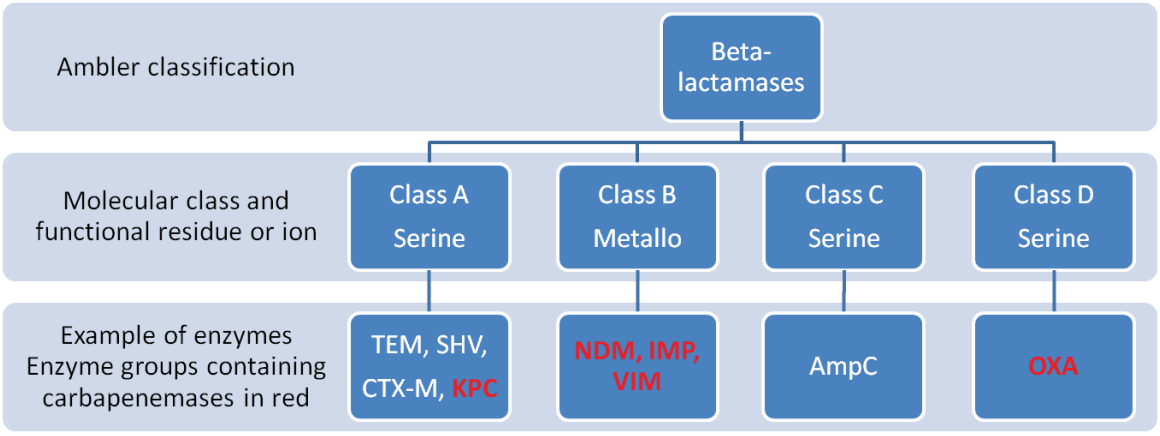


Figure 7.
The Ambler classification of beta-lactamases. Carbapenemases are found in three different groups limiting the clinical relevance of this classification.

Extended-spectrum beta-lactamase (ESBL)

The term "Extended broad-spectrum beta-lactamases" was coined by Jarlier and co-workers in 1988 to describe enzymes conferring (transferable) resistance to newer beta-lactam agents (extended spectrum cephalosporins; third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime) in opposition to the broad spectrum enzymes – mainly TEM-1 – which could hydrolyze penicillins and broad spectrum penicillins, e.g., ampicillin[23]. Later the abbreviation "ESBL" has become common. The first description of these enzymes was given by Knothe in 1983 and the clinical use of the term has recently been re-defined by Giske et al. (Figure 8)[24,25]. ESBLs as defined by Giske et al. include all acquired beta-lactamases with activity against extended-spectrum cephalosporins and/or carbapenems. Furthermore, the ESBLs are subdivided into three different groups. $ESBL_A$ consists of the classical (mostly Ambler class A) ESBLs which are inhibited by clavulanate and are characterized by their ability to hydrolyze oxyimino-cephalosporins and monobactams, but not cephamycins or carbapenems[26]. These enzymes are the main focus of this thesis. The two other groups are $ESBL_M$ (for "miscellaneous"), of which transferable AmpC is an example and $ESBL_{CARBA}$ (for

"carbapenemase"), of which *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM) and oxacillinase-48 (Oxa-48), Verona integron-encoded metallo-beta-lactamase (VIM) and imipenem metallo-beta-lactamase (IMP) are prominent examples. In this thesis the term "ESBL" refers to $ESBL_A$ unless otherwise specified.

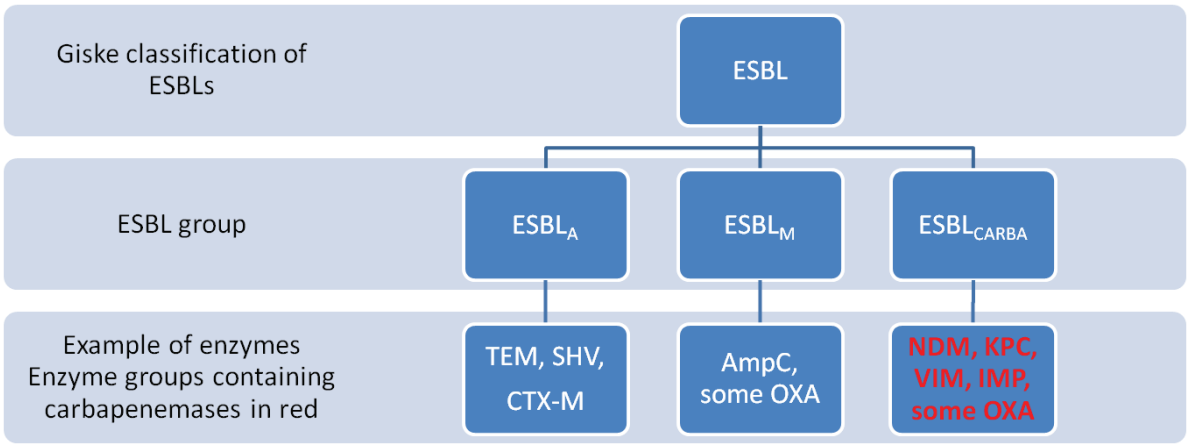


Figure 8.
The classification of ESBL enzymes. The new term $ESBL_{CARBA}$ is useful in clinical practice as infection control measures are often needed in hospitals when these are encountered.

The most important groups of $ESBL_A$ enzymes are the *Tem*oniera- (TEM), S*ulfhydryl variable* (SHV) and Cefotaximase Munich (CTX-M) enzymes, though not all the TEMs and SHVs are ESBLs. The enzyme group names reflect their historical background. The term "TEM" is coined after the patient from which the first enzyme of this group was identified in 1963 (*Tem*oniera, a Greek patient)[27,28]. The first SHV enzyme was described in 1972 by Pitton, but the name was termed after the "*sulfhydryl variable*" in 1979 because of its reaction to inhibition by the sulfhydryl group reagent p-chloromercuribenzoate[28-30]. The CTX-M enzymes were discovered in the late 1980s and are named by their preferred substrate at that time (cefotaxime) and the city where they initially were described (Munich)[31].

TEM, SHV and CTX-M beta-lactamases – molecular evolution

The original TEM and SHV beta-lactamases are very efficient and the rate of hydrolysis of their preferred substrate (aminopenicillins) is diffusion limited, i.e., the (diffusion) speed of the substrate to the active site of the enzyme limits the speed of the reaction. They may therefore be said to have reached catalytic perfection[12]. They have an ancient history and evolved long before the clinical introduction of antibiotics and even before humans. Structure-

based phylogeny indicates that the TEM and SHV group diverged from each other 3-400 million years ago[20]. In the antibiotic era, the modern TEM and SHV evolved the ability to hydrolyze cephalosporins and thus became ESBLs through point mutations[20].

In contrast to TEM and SHV, the CTX-M enzymes are a much more heterogeneous family of enzymes. They are divided into six distinct groups (groups 1, 2, 8, 9, 25 and 45) and structure-based phylogeny indicates that they diverged from a common ancestor 2-300 million years ago[20]. In the pre-antibiotic era, these enzymes were probably located on chromosomes of different *Kluyvera spp.,* and it was their mobilization to plasmids, an occurrence that presumably took place several times during the 1980s, that made them clinical relevant[26,31-34]. Their later evolution through punctual mutations has probably been driven by antimicrobial pressure in a landscape with different cephalosporins[26,35]. This has led to today's situation where approximately 60% of CTX-Ms confer resistance to ceftazidime *and* cefotaxime whilst early enzymes had low affinity for the former drug[26]. Worryingly, laboratory experiments suggest that evolutionary stasis has not yet been reached and that new variants of CTX-M enzymes, including inhibitor resistant variants, may be selected in the future[26,36].

At present, 213 TEM variants, 180 SHV variants and 144 CTX-M variants have been described (http://www.lahey.org/studies/, accessed February 10, 2014). The large majority of these are ESBLs.

Clinical history of the TEM, SHV and CTX-M ESBLs

When third generation cephalosporins were introduced in the early 1980s, they represented a major breakthrough because they could not be hydrolyzed by the broad spectrum penicillinases TEM-1 and SHV-1[37]. This introduction also marked the starting point for a rapid evolution of TEM and SHV, and enzymes that were able to hydrolyze third generation cephalosporins sprung out of both groups[38]. During the 1980s and 1990s a large number of these variants were the dominant ESBLs in clinical practice[26,37]. In this time period most patients acquired their ESBL-producing bacteria in hospitals and *K. pneumoniae* was the most prevalent species. In France approximately 30% of nosocomially acquired *K. pneumoniae* isolates were ESBL-producing in the early 1990s[39].

The first CTX-M was described in 1987. These extraordinary successful enzymes have to a large degree replaced the older TEM and SHVs in the healthcare setting and have also become important in community acquired infections[26,31,34]. They have also become much

more prevalent in *E. coli* than TEM/SHV- ESBLs. This has probably been a key to their dissemination outside hospitals and will be discussed in detail later. Their rise to predominance has been divided into three periods[26]:

i) The emergence of the different CTX-M beta-lactamases until the mid 1990s

ii) The emergence of the most widespread CTX-M enzymes from the mid 1990s to around 2000

iii) The globalization and universal dispersion of CTX-M into the community after the year 2000. This third phase coincides with the release of third generation cephalosporins from patents and their introduction as inexpensive and oral generic antibiotics.

The clinical implications of the dissemination of ESBL will be discussed later.

Prevalence of ESBL

The worldwide prevalence of intestinal carriage of ESBL has recently been described in a review by Woerther and co-workers[40]. Carriage data are sparse and this is especially true in areas where other data suggest a high carriage rate in the population. Nevertheless, Woerther et al. reported that carriage rates have increased significantly from 2002 to 2011 in all regions of the world and that the increase in the WHO region of South East Asia was most pronounced with 7.2% per year in this period (Table 1).

WHO region	Yearly increase in ESBL-E carriage rate (%)	95% Confidence interval (%)	Estimated carriage rate 2011 (%)
Africa	1.1	-0.4-2.7	23
America	0.1	-0.6-0.9	5
Eastern Mediterranean	3.5	2.0-4.9	35
Europe	0.5	0.04-0.9	5
South East Asia	7.2	5.1-9.2	70
Western Pacific	1.5	0.04-2.9	25

Table 1.
The increase in intestinal carriage of ESBL-producing Enterobacteriaceae (ESBL-E) in the general population in the six WHO-regions from 2002-2011[40]

As many as 1-2 billion people are probably intestinal carriers of ESBL-producing Enterobacteriaceae (ESBL-E). Thus, the spread of ESBL has been said to have reached "pandemic" proportions[40,41].

In parallel with increased intestinal carriage we have seen an increased prevalence of infections caused by ESBL-E. This is illustrated by the European and Norwegian data of *E. coli* from urinary- and invasive infections that were resistant to 3rd generation cephalosporins in 2002 and 2011 (Figure 9 and Table 2)[40]. *E. coli* is the most important ESBL-producing pathogen and ESBL-production caused 85-100% of the resistance to 3rd generation cephalosporins in the European material[9,40].

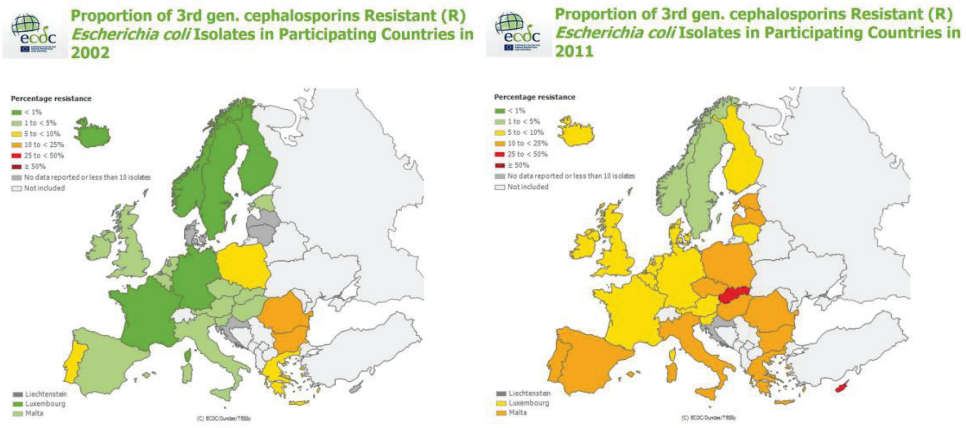


Figure 9.
Proportion of 3rd generation cephalosporins resistant *E. coli* isolates in 2002 and 2011 in Europe. Green colours represent a low proportion while yellow, orange and red represent higher proportions.
(Thanks to the European Centre for Disease Prevention and Control (ECDC) 2005 – 2013, figures free for non-commercial use)

Country	Proportion of 3rd generation cephalosporins resistant *E. coli* isolates (%)		Yearly increase (%)
	2002	2011	
Greece	1	15	1.4
Poland	0	12	1.3
Portugal	0	11	1.2
Italy	0	20	2.0
United Kingdom	0	10	1.1
Hungary	1	15	1.5
Slovakia	0	31	3.0
Spain	1	12	1.2
Netherlands	0	6	0.5
Germany	1	8	0.7
France	1	8	0.7
Finland	0	5	0.5
Sweden	0	3	0.3
Norway	1	4	0.3
Iceland	0	6	0.6
Romania	0	21	2.1
Total (non-weighted)	**1**	**12**	**1.2**

Table 2.
The proportion of 3rd generation cephalosporins resistant invasive *E. coli* isolates in some European countries from 2002 to 2011 with yearly increase. 85-100% of this resistance was caused by the presence of ESBL-genes. Data from EARS-Net.

The prevalence in Norway is low compared to other countries, but we have nevertheless observed a 10-fold increase between 2002 and 2012 (Figure 10).

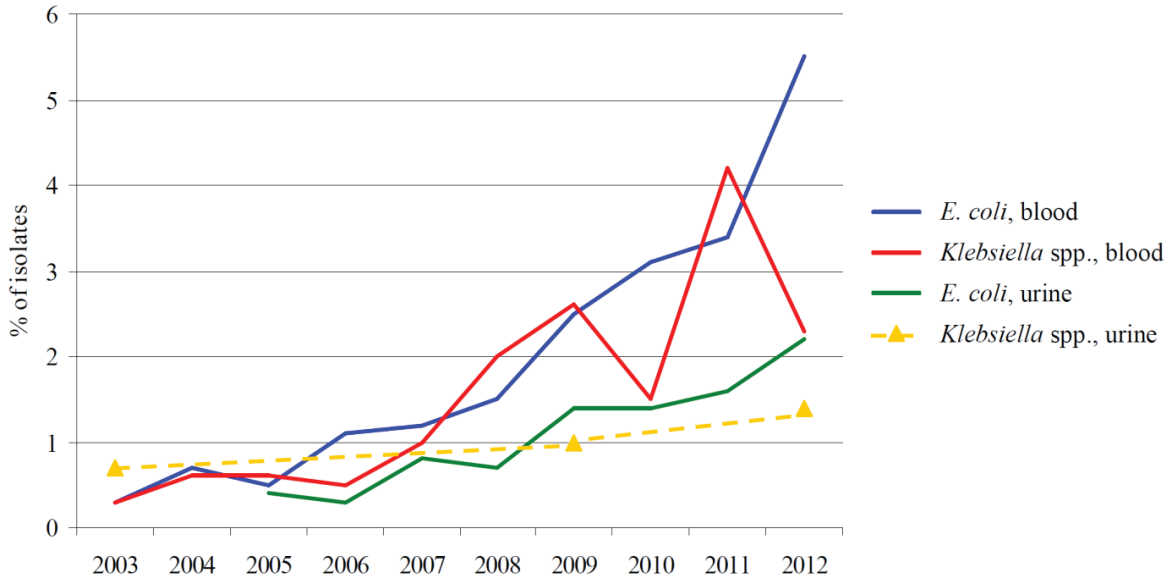


Figure 10.
Prevalence of ESBL-production among *E. coli* and *Klebsiella spp*. from blood and urine 2003-2012 in Norway[19].

The importance of *E. coli* in the dissemination of ESBL

In contrast to before the year 2000 when *K. pneumoniae* was the dominant bacteria harbouring ESBL, the clinical bacterial species most often harbouring ESBL at present is *E. coli*. The introduction of CTX-M-type ESBLs in the *E. coli* population has been extensive and is probably a result of several factors including successful combinations of *E. coli* clones with genetic machinery consisting of insertion sequences, integrons and transposons on plasmids that are well-adapted for horizontal gene transfer and maintenance in this species[26]. The ESBL-genes are frequently harboured on multiple antibiotic resistance plasmids and co-selection further favours their spread in antibiotic exposed environments. One example of a successful relationship between an *E. coli* and a plasmid is in the *E. coli* ST-131 strain, which is a pandemic sequence type (ST) of virulent *E. coli* often causing extra-intestinal infections. *E. coli* ST-131 often, but not always, harbours a plasmid coding for the CTX-M 15 gene. Early evidence suggested that ST-131 strains already present in the environment obtained ESBL-genes through multiple horizontal gene transfer events and this is probably an important mechanism for how this combination of ST-type and resistance mechanism has emerged[40,42].

However, one newer study based on full-genome sequencing of a diverse collection of *E. coli* isolates from The United States and Germany also pointed to a prominent role of clonal expansion of particularly virulent and multiple antibiotic resistant subclones in these countries[43]. In this collection one fluoroquinolone resistant non-ESBL-producing subclone accounted for 58 of 61 (95%) fluoroquinolone resistant *E. coli* ST-131 while a subclone nested within this subclone harboured 20 of 22 (91%) fluoroquinolone resistant and CTX-M 15 producing *E. coli* ST-131. This is a rapidly developing field and larger studies from more countries will probably further elucidate whether frequent horizontal gene transfer events or clonal expansion is most important for the dissemination of ESBL in the *E. coli* population. *E. coli* is highly prevalent in the human population and is a leading cause of bacterial disease. It is the causative agent in more than 75% of UTIs and is the most commonly encountered species in blood stream infections (BSI) in Norway, representing more than 30% of positive blood cultures[19,44]. Therefore ESBL now affects one of the most important human pathogens. Another cause for concern is the natural habitat of *E. coli*. *E. coli* is the primary commensal facultative bacteria in the human intestinal tract, but its natural habitat extends to the intestinal flora of wildlife, companion animals and livestock and watery natural habitats including creeks and rivers, sediments, lakes and the sea[45,46]. It is estimated that only half of the *E. coli* population is intestinally located[45]. The dispersal of resistant *E. coli* from human waste, sewage and agriculture into natural watery environments is vast and many of these are continuously contaminated by resistant *E. coli*[40,47-55]. The importance of these environments in the dissemination of ESBL will be discussed in the following chapter.

Modes of dissemination

The spread of CTX-M may be compared to the spread of TEM-1 from the 1960s to today[56]. The rate of aminopenicillin resistance in invasive *E. coli* isolates in Europe is currently 57.4% whereby TEM-1 accounts for up to 60%. This illustrates the vast potential of a successful resistance gene in the right environment and shows that for the TEM-1 gene Europe has been very favourable[9]. We have experienced that for the CTX-M genes, not only Europe, but the whole world seems to be an advantageous environment.

A cycle of transmission - summary of research into transmission

Cycles of transmission of bacteria, plasmids and other mobile genetic elements containing CTX-M between individual hosts, human- and animal populations and the environment and

back to the human population probably exist (Figure 11). These cycles are reinforced by selection and horizontal gene transfer occurring in the different compartments. Results from research into each of these compartments will be presented separately.

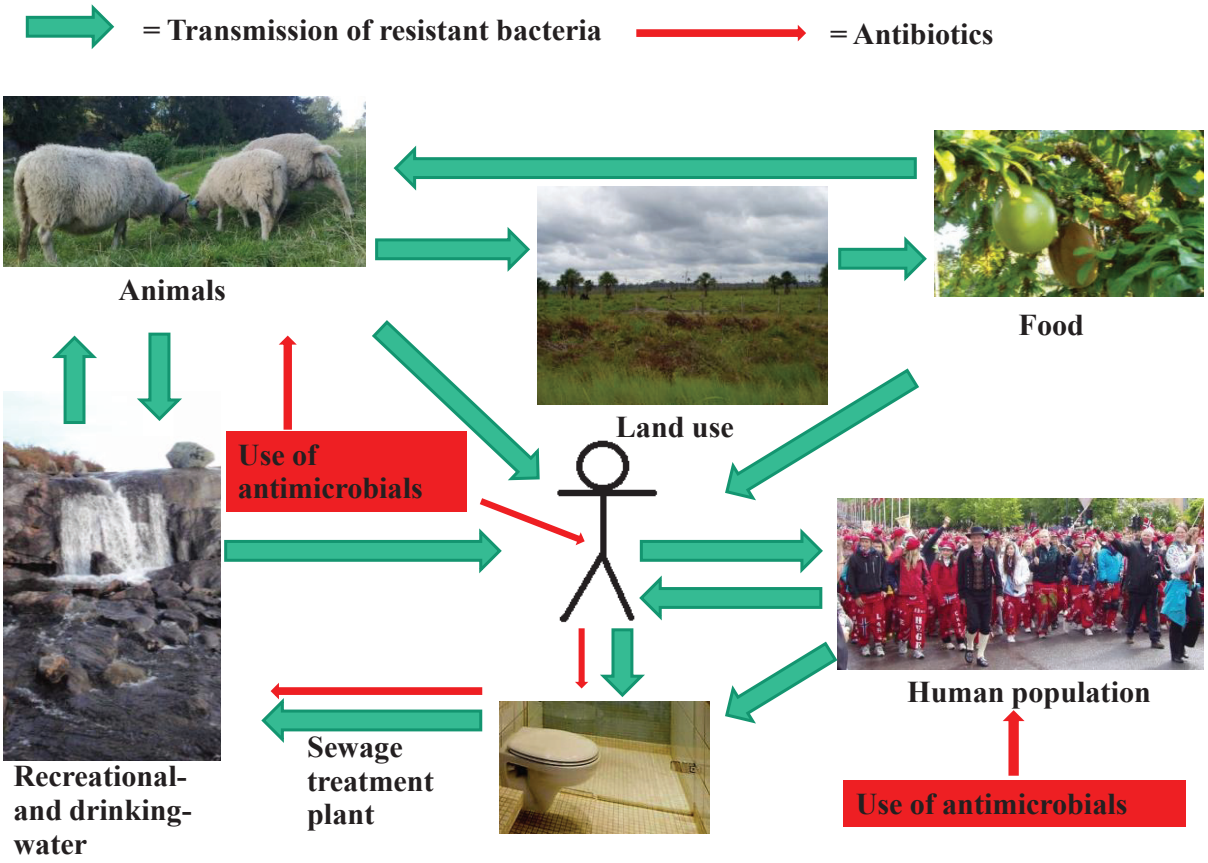


Figure 11.
Dissemination of ESBL between the patient, the population and the environment.

Intestinal carriage

Intestinal carriage of ESBL has been reported to be of long duration. Moreover, even transient bacteria carrying ESBL plasmids may donate their resistance genes to a host through horizontal gene transfer in the gut[57-60]. Faecal carriage is the source of faecal-oral transmission and also pollution into the environment and therefore plays a key role in the cycle of transmission of ESBL.

The environment and food are possible sources of intestinal carriage and will be presented separately, but an interesting point regarding this deserves to be mentioned: In a Swedish study intestinal ESBL colonization rates before and after foreign travel was

investigated. Travel to countries with a high rate of ESBL carriage in the population was associated with a higher rate of carriage when returning home than travel to countries with a low rate of ESBL[40,61-63]. This shows that intestinal ESBLs may be acquired quite readily probably through faecal-oral exposure.

The transfer of ESBL within families has been studied and pulsed-field gel electrophoresis has revealed that strains of ESBL in household members are frequently similar. The ESBL carriage rate among household members of known carriers is also higher than in the normal population. These findings support, but does not confirm, intra-household transfer[40,64,65].

Food chain

Another important field of research is the possible transfer of ESBL from food to humans. This is a hot-debated topic, but several studies have documented the presence of ESBL in food and even tap water[66-68]. At least one study has shown a genetic relationship between ESBL-genes found in food, animals and in humans[66]. However, evidence also suggests that ESBL-E of human and animal origin usually are different[69]. It has been suggested that intestinal carriage of ESBLs of animal origin may be an intermediate step between food and infection[66]. The spread of ESBL among farm animals may be exacerbated by the use of antimicrobials that select for ESBL-producing bacteria in food production[70,71]. Unfortunately, co-resistance is common in ESBL-producing bacteria and therefore not only beta-lactams, but also most other groups of antimicrobials, might enhance selection of these strains in a food production setting.

One question raised by research into transmission of ESBL is whether presence of ESBL-genes in food is acceptable and whether antimicrobials selecting for ESBL-genes should be allowed in farm animals. A precautionary approach to these questions would be to outlaw ESBL and certain antimicrobials in food and food production. The growth promoter avoparcin, with a vancomycin-like antimicrobial property was withdrawn from the marked because of selection of vancomycin resistant enterococci, a potential human health hazard[72,73]. To my knowledge this has not been considered in the case of ESBL.

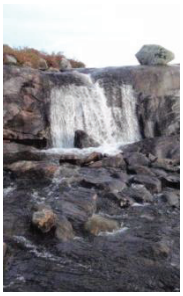
Horizontal gene transfer in the intestinal tract and sewage

The process of horizontal gene transfer is considered important in the dissemination of genes related to antimicrobial resistance. The

intestinal tract of humans and animals is one location where this process has been shown to occur. In one experiment human volunteers were given a tetracycline resistant *E. coli* of animal origin during tetracycline treatment and transfer of the tetracycline resistance to indigenous *E. coli* was observed[74]. Another study, also in human volunteers, but without antibiotic treatment, demonstrated *in vivo* transfer of the vanA gene conferring vancomycin resistance between a donor and a recipient strain of *Enterococcus faecium*[75]. Furthermore, a recent study by Titelman and co-workers found that long term carriage of ESBL was associated with finding the original ESBL-gene in another strain or species than early in the carriage episode[76]. This suggests that the ESBL determinant achieves long term intestinal presence through transfer into indigenous strains, and thereby increases its chances of transfer to another host.

Another habitat where horizontal gene transfer is occurring is in wastewater treatment plants[77,78]. These plants process big volumes of wastewater containing high densities of bacteria. In one study horizontal gene transfer occurred approximately 100 times more frequently when donor and recipient strains were mixed into sterile wastewater than sterile water from a lake and incubated for 24 hours at 35 ºC. Thus, these plants may facilitate dissemination and persistence of large quantities of resistance genes through horizontal gene transfer[79].

Environment



The environmental reservoir of ESBL is huge, and ESBL has been found in most rivers below 1000 m altitude in Switzerland and recently in soil samples from France[55,80]. The soil had been fertilized with manure one year before sampling, suggesting the possibility of long term survival of *E. coli* after such disposal of animal waste. Both living bacteria and intact DNA survive ordinary sewage treatment[77]. ESBL-E has been identified in the affluent and effluent of sewage treatment plants and thus, these plants are sources of environmental pollution of ESBL[47,54,81-84]. Even surviving ESBL-genes represent a source of pollution from these plants as they may be taken up by bacteria by natural transformation or other modes of horizontal gene transfer. Bacteria released into the environment or transformed bacteria may end up in humans either by direct transfer through exposure to environmental waters or dust or indirectly through the food chain or animals[48-52,85,86].

During the last few years, it has been recognized that antibiotics themselves might be considered a class of water contaminants[87]. They are not only applying a selective pressure

favouring bacteria harbouring resistance determinants in sewage treatment plants, but are also to a large degree released from these plants into the environment, applying a selective pressure even there[87,88]. This has been the focus of a recent review by Hughes et al. demonstrating the importance of very low concentrations of antibiotics in selection of resistant phenotypes[89]. This further reinforces antibiotics´ place as significant pollutants.

The largely unchecked dispersion of antimicrobial resistant bacteria, resistance genes and antibiotics into the environment may have a surprising positive consequence. In his review Dodd points out that the efficacy of destruction of antibiotic resistance genes and resistant bacteria varies between different treatment methods[77]. Thus, selecting the best method of water treatment may reduce the spread of resistant bacteria. These engineered aquatic systems may contribute to the spread of resistance today, but may also represent a key to reduce the problem in the future.

The One Health Initiative

To counteract emission of antimicrobials and transmission of resistant microbes between humans, animals and the environment, a holistic and multidisciplinary perspective is needed. This is encapsulated in the "One Health/One Medicine" perspective which has gained significant attention during the past years and is encapsulated in the "One Health Initiative"[90,91]. Newer research into antimicrobial resistance often directly or indirectly draws ideas and inspiration from this approach.

Clinical implications

The natural habitat in the body for most ESBL-producing bacteria is the gastrointestinal tract and most infections caused by these bacteria originate from this organ or occur in nearby organs such as the urinary- or biliary tract. A large majority of these infections are also caused by Enterobacteriaceae (ESBL-E). The clinical problems caused by ESBL-production can be divided into immediate problems in the choice of appropriate treatment and longer term problems of increased antimicrobial resistance.

Treatment problems

Infections caused by ESBL-E are important in clinical practice due to their association with increased morbidity and mortality[92]. The reason for this is not that ESBL-Es are impossible to treat, but because they often are resistant to the initial empirical therapy used. This has been

explored in a recent systematic review by Rottier et al.[93]. The authors found a pooled odds ratio (OR) of 2.4 (95% confidence interval (CI): 1.9-2.9) for mortality due to BSI caused by an ESBL-E compared to a non-ESBL-E in 32 studies. The data indicated that this excess mortality was partly mediated through inadequate empirical therapy. As ESBL-Es are resistant to most beta-lactam antibiotics and often exhibit co-resistance, this is an expected finding. Some of the studies included the OR after adjustment for inadequate empirical therapy and the adjusted OR was only 1.4 (CI 1.04-1.8) in these studies. This indicates that other factors related to ESBL-E or patients infected with ESBL-E also contribute to the excess mortality.

The treatment problems in infections caused by ESBL-E are also related to co-resistance. Often co-resistance is a result of the ESBL plasmid harbouring several antibiotic resistance determinants. This makes treatment more difficult and leads to co-selection of several resistance determinants. Thus, not only cephalosporins and penicillins, but also other antibiotics may select for ESBL-producing strains. Table 3 shows the resistance pattern in ESBL-producing and non-ESBL-producing strains in 1646 *E. coli* blood culture isolates from Norway in 2012. The resistance rates to non-beta-lactam antibiotics are three to eleven times higher in the ESBL-producing strains[19].

Antibiotic	**ESBL-producing *E. coli* (n=90) resistance (%)**	**Non-ESBL-producing E. *coli* (n=1556) resistance (%)**
Trimethoprim-sulfamethoxazole	82	24
Ciprofloxacin	81	7
Gentamicin	41	4

Table 3.
Antimicrobial resistance in percent in ESBL-producing and in non-ESBL-producing *E. coli* in blood cultures in Norway, 2012[19].

When the susceptibility and ESBL-status of the infecting strain are known, the choice of treatment is usually straightforward. Often a carbapenem or a beta-lactam/beta-lactamase inhibitor combination is chosen in a hospital setting, but most strains are susceptible to several different antibiotics[94]. Unfortunately, alternative antibiotics are often not as efficient as the cephalosporins in life threatening infections or have contraindications that make their use difficult. Furthermore, bacterial identification and susceptibility testing takes 1-3 days in most laboratories and in the meantime most patients will receive the empirical treatment. Failure of empirical treatment due to ESBL-production is probably the most important direct clinical

consequence of ESBL-E. As outlined in the next section, this may also force the use of broad spectrum empirical treatment regimens to cover ESBL and associated resistance.

Increased antimicrobial resistance

Increased prevalence of antimicrobial resistance like that represented by the spread of ESBL-E will lead to increased use of broader spectrum antimicrobials with negative ecological consequences. Empirical treatment must cover a majority of the most likely microbes and must be adjusted to the local levels of antimicrobial resistance. Thus, in areas with a high ESBL prevalence empirical treatment will have to cover ESBL-E. This most often means increased use of carbapenems or beta-lactam/beta-lactamase inhibitor combinations. The use of these very broad spectrum antibiotics will also increase because they are employed in the definite treatment of ESBL-E. Together, this creates a vicious circle, where an increase in the prevalence of antibiotic resistant bacteria leads to increasing use of (broad spectrum) antibiotics which again leads to the development of increased antimicrobial resistance. Several authors foresee that the next turn in this spiral will be increased development of resistance against the carbapenems through $ESBL_{CARBA}$ like the KPC enzymes most prevalent in the United States, Israel and Greece and the NDM enzymes most prevalent in the Indian subcontinent. Some disquieting reports from India indicate a local NDM carriage rate up to18% [95-97]. The Indian subcontinent is an area where the spiral of increased resistance seems to turn particularly fast, possibly due to low hygienic standards combined with extensive use and availability of broad spectrum antibiotics like carbapenems as illustrated in Figure 12. For other countries the situation here may serve as a warning of what may go wrong if one does not take action against antibiotic resistance.

Finally, the awareness regarding treatment of ESBL-E may also represent an opportunity to counteract the upwards spiral of antimicrobial resistance. This is because ESBL-E frequently is susceptible to narrow spectrum antibiotics – at least *in vitro*. Increased use of these, especially for selected infections such as UTIs, may allow efficient treatment without large negative ecological consequences. This requires awareness of the problem, timely availability of antimicrobial susceptibility testing results and desirably also risk stratification of patients before empirical treatment. For some narrow spectrum beta-lactam antibiotics this also requires research into the *in vivo* effect of these against ESBL-producers.

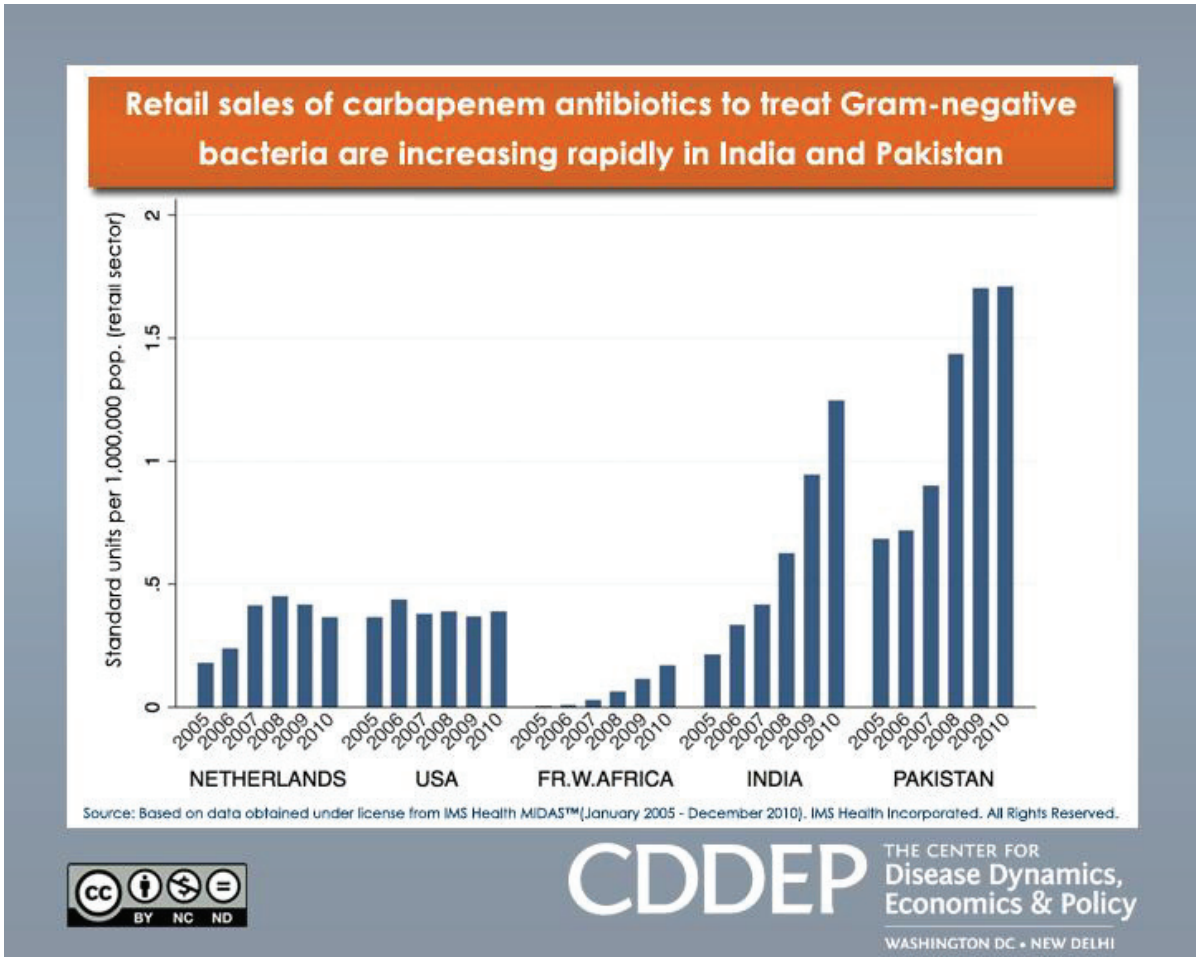


Figure 12.
Retail sales of carbapenem antibiotics to treat gram-negative bacteria are increasing rapidly in India and Pakistan. (Creative Commons Attribution-NonCommercial-No Derivs 3.0 Unported License, thanks to The Centre for Disease Dynamics, Economics & Policy, http://www.cddep.org/tools/retail_sales_carbapenem_antibiotics_treat_gram_negative_bacteria_are_increasing_rapidly_india_)

Treatment of UTI caused by ESBL-E

International treatment guidelines by Gupta et al. (2011) endorse with an A1-grading of recommendation, the use of mecillinam, nitrofurantoin, fosfomycin and trimethoprim-sulfamethoxazole as a first choice empirical treatment of uncomplicated lower UTI in women[44]. In cases with pyelonephritis or suspected pyelonephritis, oral ciprofloxacin or intravenous aminoglycoside/3rd generation cephalosporin/carbapenem are recommended depending on the patient's clinical status and local susceptibility data. The ecological impact of the treatment of UTI has carefully been considered in the work with these guidelines because UTI are among the most common infectious diseases. Thus, antimicrobials used to treat UTI have a large impact both on the total human antimicrobial consumption and on the

antimicrobial selective pressure in the population as a whole. This is illustrated by data from Norway, showing that more than 3.8 million DDD of three antimicrobials commonly used in UTI (pivmecillinam, trimethoprim and nitrofurantoin) were prescribed in 2012 in a country populated by approximately 5 million inhabitants[98].

If a UTI is caused by an ESBL-E or in a high prevalence setting, narrow spectrum alternatives for treatment may be lacking. Mecillinam is one of the drugs which may have a place in this setting, but it's *in vivo* efficacy against ESBL-producing *E. coli* has been questioned[99].

Mecillinam

Mecillinam is an amidinopenicillin with selective activity against gram-negative bacteria and Enterobacteriaceae in particular. It is widely used in the Scandinavian countries. *In vitro* data and some *in vivo* studies suggest that it has a useful activity against ESBL-E[95,100-106]. Some of the drugs key characteristics will be presented in the following.

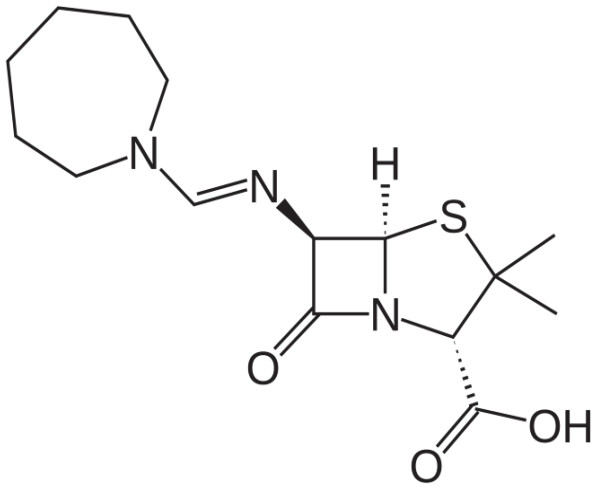


Figure 13.
The mecillinam (Amdinocillin) molecule.

Pharmacodynamic

Because mecillinam is a beta-lactam (Figure 13), it is assumed that the time above minimum inhibitory concentration (MIC), which should be >40%-50%, is the key parameter determining its clinical efficacy[107]. Mecillinam can be administered by parenteral route or orally as a prodrug, the pivaloyloxymethyl ester pivmecillinam, which after absorption is converted to the antibacterial active mecillinam[107]. Roholt has presented the pharmacokinetic properties of

mecillinam and pivmecillinam[108]. The serum half-life is approximately 30 minutes. Animal data on the distribution one hour after intravenous administration of 50 mg/kg body weight resulted in similar distributions in dogs, rats and rabbits. The data from dogs showed a very high concentration in urine (20,726 mg/L), but also in the bile (1,427 mg/L), the kidney (119 mg/L) and the liver (114 mg/L) the concentrations were higher than in serum (34 mg/L). The lowest concentrations were found in the brain (0.6 mg/L), the medulla (1.4 mg/L) and the eye (3.9 mg/L). Roholt also found that the oral availability of pivmecillinam was high and independent of food in the stomach[108]. The very high urinary concentrations obtained have been thoroughly documented and are important regarding its use in UTIs[109]. In accordance with this, the main indication for use of mecillinam is uncomplicated UTIs. Its use against other infections is more controversial and MIC breakpoints have not been defined in these circumstances. In Roholt`s experiments intravenous injection of 400 mg mecillinam in nine volunteers resulted in a maximal serum concentration of 28±5 mg/L. After one hour the mean serum concentration was 5 mg/L. When administered orally as pivmecillinam 273 mg of mecillinam (corresponding to 400 mg pivmecillinam) gave a maximal serum concentration of 5.2±1 mg/L which was obtained at 53±8 minutes. One hour later the mean serum concentration was still 3 mg/L[108]. For comparison the ECOFF value of mecillinam is 1 mg/L in *E. coli* and the cefotaxime serum concentration one hour after *infusion* of 1000 mg was 8 mg/L[110].

Excretion

Mecillinam is broken down into several metabolites in the body, both active and inactive[108]. These metabolites are also excreted in the urine and bile. Experiments have shown that about half a given dose is excreted as biologically active substance in the urine. In dogs, 3.1% was excreted as active substance in the bile. Roholt refers to experiments in dogs with ^{14}C-labelled pivmecillinam hydrochloride administered orally in which 44% of the ^{14}C-dose was excreted to the faeces. However, Sullivan et al. had difficulty detecting mecillinam in faecal samples in their study citing its liability in body fluids or faecal beta-lactamases as possible causes[111]. This is important because excretion into the gastrointestinal tract may select for mecillinam resistant bacteria here. Mecillinams ecological profile and development of resistance will be discussed later.

Mode of action

Like other beta-lactams mecillinam acts on a PBP and its target in *E. coli* is PBP2. PBP2 is responsible for the lateral growth of *E. coli* and is present in the cylindrical part of the envelope and the constriction site during cell division[112].

The classification of PBPs has historically been based on gel electrophoresis rather than molecular structures and sequences[113]. Thus, PBP2 in *E. coli* is structurally related to for instance PBP3 in *S. aureus* and PBP1 in *Chlamydia trachomatis.* The newer classification of PBPs is more functionally relevant and this is nicely exemplified by a paper showing that mecillinam has an activity against *C. trachomatis* and that this is mediated through PBP1 (which corresponds to mecillinam`s target in *E. coli*; PBP2)[14,113]. As *C. trachomatis* is thought to lack a peptidoglycan cell wall, this supports the notion that PBPs may have other essential functions in bacteria that may be inhibited by beta-lactams.

The action of mecillinam on *E. coli* was studied microscopically in 1975 by Tybring et al[114]. They found that *E. coli* developed spherical shapes under the influence of mecillinam and that mecillinam had a time-dependent bactericidal activity. More recently this has been elaborated on a molecular level by Den Blaauwen et al. who also found that PBP2 in *E. coli* is essential for maintaining the diameter of the cells[115].

The *E. coli* PBP2-like selectivity of mecillinam contrasts other beta-lactams in clinical use which have a higher affinity for other PBPs. Intuitively this opens for synergy between mecillinam and other beta-lactams and indeed this has been described. In a 1976 study by Grundberg et al. synergy between mecillinam and amoxicillin was observed in 15 of 20 strains of gram-negative bacteria in an *in vivo* mouse model using peritoneal infection[116].

Ecology and resistance development

Mecillinam is mostly targeting gram-negatives and its presence in the intestines, which can select for resistant strains, is important as this may affect human health. Sullivan et al. have reported a relatively minor ecological impact of pivmecillinam (200-400 mg 2-3 times daily for 7 days) on the vaginal, skin, oropharyngeal and intestinal human microflora[111,117]. The most profound change was a >99% reduction in intestinal *E. coli,* which corresponds to its quite selective activity against Enterobacteriaceae and also to excretion of active drug in faeces. Although the authors did find mecillinam in a low proportion of faecal samples in the study, they argue that the ecological profile is favourable compared to most other alternative drugs. A favourable ecological profile is also indirectly supported by the observed stable and

low (<5%) rate of resistance to mecillinam in uropathogenic *E. coli* in several surveys including the Scandinavian countries with widespread use of pivmecillinam over many years[118,119]. Furthermore, Poulsen et al. showed no evidence of clonal spread of mecillinam resistant bacteria indicating a low propensity for such spread[120]. The true impact of mecillinam on the total intestinal flora would probably be further elucidated if metagenomic analysis of the gut microbiome before and after treatment were performed, but so far mecillinam`s ecological properties seem favourable compared to alternatives like ciprofloxacin or amoxillin.

Mecillinam is established as a first choice antimicrobial against uncomplicated UTI in countries where the drug is available. From an ecological perspective availability in more countries is desirable.

The use of mecillinam is not without perils, though. Hossain et al. found a prevalence of mecillinam resistance of up to 17% in *Shigella spp* in Bangladesh in the 1990s[121]. In this area mecillinam was widely available and used in the treatment of *Shigella* infections. Thus, the importance of prudent use is valid for mecillinam as for other antimicrobials.

Use in relation to beta-lactamases

Use of mecillinam in infections caused by ESBL-producers (both $ESBL_A$ and $ESBL_{CARBA}$) is not yet established. The MICs of mecillinam in $ESBL_A$ producing strains seem to be inoculum dependent[99]. Nevertheless, $ESBL_A$ producers are usually *in vitro* sensitive and if this is confirmed *in vivo* mecillinam can become an alternative in the treatment of UTI in many countries with a high prevalence of these bacteria[95,100-106]. India and Pakistan are two such countries where mecillinam is allegedly not available[122]. Moreover, Perry et al. found only 5% mecillinam resistance among NDM-1 producing *E. coli* in Pakistan making the introduction of the drug to these countries even more attractive[95].

Focus areas of this thesis

This thesis focuses on three key areas related to infection with, and spread of ESBL producing bacteria.

The first area concerns risk factors for infections caused by these bacteria. The knowledge of risk factors is one step towards identifying the underlying causes of a disease. This is important if one wants to design interventions to prevent or limit its spread. In some situations, like when instituting empirical antimicrobial treatment, it might also be of interest to know which patients who are at the highest risk of having an ESBL-positive infection. We

have concentrated our attention on *E. coli* and *K. pneumoniae* with the "classical" $ESBL_A$ beta-lactamases CTX-M, SHV and TEM. Furthermore, we have focused on CA-UTI because this is the most prevalent infection caused by these bacteria. CA-UTI may also be considered a surrogate marker for the acquisition of ESBL-E. Risk factors connected to the health care system regarding these infections have previously been explored in depth, whilst risk factors for the community acquired infections that have recently increased to "pandemic" proportions, have in contrast been much less examined. We have therefore studied community acquired infections exclusively.

The second area of investigation is carriage of ESBL-E. This is important because of its long duration that increases the period of transmissibility. The existence of intestinal carriage is well described, but to our knowledge carriage in the oral cavity, the first part of the gastrointestinal tract, has not yet been investigated. Oral carriage might both prolong intestinal carriage and may also theoretically increase transmissibility due to possible spread from the oral cavity directly or indirectly to other people.

Finally, the last area investigated is mecillinam treatment of CA-UTI caused by ESBL-producing *E. coli*. Such infections are increasingly common and alternative drugs for empirical and definite treatment are in demand. Increased use of mecillinam and other narrow spectrum antimicrobials as alternatives to broader spectrum drugs like fluoroquinolones, cephalosporins and carbapenems, is desirable since it might reduce the selective pressure that promotes the rise of antimicrobial resistant bacteria.

Aims of the thesis

Paper I – The case-control study

To test the hypothesis that the risk for CA-UTI caused by ESBL-producing bacteria is influenced by lifestyle associated factors and antimicrobial use.

Paper II – The oral carriage study

To test the hypothesis that supragingival plaques may be colonized by ESBL-producing bacteria in intestinal carriers of these bacteria.

Paper III – The mecillinam study

To test the hypothesis that mecillinam has a useful clinical effect in the treatment of CA-UTI caused by ESBL-producing *E. coli* and thereby explore its possible use as empirical therapy against CA-UTI in settings with a high ESBL prevalence.

Material and Methods

Background

This thesis is a part of the "Extended Spectrum beta-lactamases – Carriage, environmental dissemination And Population Epidemiology" ("ESCAPE") project which was developed and initiated at the Department of Medical Microbiology at Bærum hospital by the author together with the supervisors Pål A. Jenum and Arnfinn Sundsfjord. The planning of this project was initiated in 2008 when the author was specialist registrar at the Department of Infectious Diseases at Bærum hospital and experienced the problems faced by patients with ESBL-E on a regular basis. Inclusion of patients began after all permissions were obtained in early 2009.

Setting

The source population for the ESCAPE project was inhabitants living in the geographical area covered by the Department of Medical Microbiology at Vestre Viken Hospital Trust. This is a mixed urban, suburban and rural area in the South-Eastern part of Norway. The department includes two laboratories analysing samples from in- and outpatients in this area which comprises four hospitals and approximately 470.000 inhabitants. The inclusion period was from February 2009 to June 2012. Before the Departments of medical microbiology at Drammen and Bærum hospital were merged in March 2010 data were only collected from Asker and Bærum communities (~180.000 inhabitants). The same was true for patients included after April 2011.

Ethics

The project and all its subprojects involving human participants have been approved by the Regional Committee for Medical and Health Research Ethics, following the Declaration of Helsinki principles (reference number: 2009/2037 BS-08901b). The part investigating treatment with mecillinam was registered in ClinicalTrials.gov (Identifier: NCT01838213).

Summary of methods

Paper I – The case-control study

In this case-control study frequency of possible risk factors for CA-UTI were compared between patients with disease caused by ESBL-producing and non-ESBL-producing strains using univariate techniques and multivariable logistic regression.

Paper II – The oral carriage study

Faecal samples from the participants of the case-control study were collected and supragingival plaque samples from a subset of patients with faecal carriage of ESBL-producing strains were taken. The oral samples were analyzed for the presence of ESBL-producing bacteria and ESBL-genes.

Paper III – The mecillinam study

Empirical treatment outcomes in patients with CA-UTI caused by ESBL-producing and non-ESBL-producing bacteria were obtained from the participants in the case-control study supplemented with some extra cases. Multivariate logistic regression was used to explore associations between treatment outcome, ESBL-status and treatment with mecillinam. The outcome of patients treated with mecillinam was also compared to the outcome of patients receiving other antimicrobials.

Participants

Inclusion and exclusion criteria

All patients ≥18 years old with a urine sample yielding *E. coli* or *K. pneumoniae* >10,000 colony forming units/ml submitted to one of our two laboratories were considered for inclusion. The following potential participants were excluded:

1. Participants who previously had been diagnosed with any ESBL-producing bacteria as registered in the laboratory's database.
2. Participants hospitalized or residing in a nursing home for >24 hours during the last 31 days.
3. Participants who had lived in Norway for less than one year.
4. Participants who were unable to answer the questionnaire.

Selection and inclusion of participants

All eligible patients with an ESBL-producing strain were invited to participate in the study as cases. For each case one to four unmatched eligible patients with non-ESBL-producing strains were consecutively evaluated for possible participation in the study as controls. These control-patients were selected using Excel® (Microsoft, Redmond, Washington) randomization. After preliminary screening, possible cases and controls were informed about the study by ordinary mail and invited to participate by returning a written consent together with a faecal sample. From February 2009 to April 2011 non-responders were contacted twice and in this period participants were contacted the same week as they submitted their urinary sample or the consecutive week. After April 2011 inclusion was managed as one batch in June 2012 and no reminder was sent to non-responders. From these groups of patients, participants for the different sub-studies were selected:

Paper I – The case-control study

Patients included in the period from February 2009 to April 2011 were included in the case-control study.

Paper II – The oral carriage study

A subgroup of patients from the case-control study who submitted an ESBL-positive faecal sample in two periods between March 2010 and November 2011 were invited to participate in this sub-study.

Paper III – The mecillinam study

Patients from the whole inclusion period were considered for participation, but only if *E. coli* was isolated from their urinary sample. Furthermore, only patients who had received antibiotic treatment for their UTI were invited. The treatment had to be instituted at the same day as the urinary sample (allowing ±1 day for uncertainty regarding the dating of the sample). Only antimicrobials appropriate for UTI (trimethoprim, trimethoprim-sulfamethoxazole, ciprofloxacin, ofloxacin, nitrofurantoin, pivmecillinam, amoxicillin or cephalexin) were considered.

Data collection

Microbiological data

Urine samples

Urine cultivation and bacterial identification were performed using ChromID CPS3 agar plates and the VITEK-2 system (both BioMerieux, Marcy l'Etoile, France). Pink to burgundy colonies on the chromogenic media were interpreted as *E. coli*, while other gram-negative colonies on the chromogenic media were identified using the VITEK-2 system (GN ID card). Antimicrobial susceptibility testing and interpretations including ESBL screening were performed using VITEK-2 (AST- N029, N122 or N209 card) which reports MIC of mecillinam in categories ≤1, 2, 4, 8, 16, 32 and ≥64 mg/L based on measurements in wells with mecillinam concentrations of 1, 3, 8 and 32 mg/L. All isolates resistant to cefpodoxime, cefotaxime or ceftazidime were selected for confirmatory ESBL testing using the E-test gradient system (AB-Biodisk, BioMerieux) and species identification for these *E. coli* strains was also confirmed using the VITEK-2 system (GN ID card). Clinical breakpoint interpretations were performed according to EUCAST[123]. The clinical breakpoint for resistance to mecillinam in *E. coli* was >8 mg/L during the study period[123].

Faecal samples (paper II)

Faecal samples were collected by the participants themselves using a sterile cotton swab applied to the toilet paper after defecation. The sample containers (Amies agar gel swabs with charcoal Sarstedt/Copan, Numbrecht, Germany) were returned by ordinary mail and cultured aerobically on an ESBL selective agar plate (ChromID ESBL, BioMerieux), on a non-selective lactose agar plate as growth control and in a selective broth containing 2.5 mg/l of cefotaxime for 16-24 hours before inoculation on a ESBL selective agar plate. Species identification on the ESBL selective agar plate was based on characteristic pink to burgundy oxidase-negative colonies for *E. coli.* Species identification of green colonies was obtained using the VITEK-2 system (GN ID card, BioMerieux).

Oral plaque samples (paper II)

Supragingival plaque samples were collected with sterile Gracey-designed steel curettes (Stalan GMBH, Ahrensburg, Germany) from the mesio-buccal aspect of every tooth. From participants with dental prostheses or implants biofilms were collected from these restorations. The samples were immediately transferred to ≈5.5 ml of a pre-reduced anaerobic sterilized

transport medium (Dental Transport Medium, Anaerobic System, Morgan Hill, California), a medium in which also facultative organisms will survive. The samples were then homogenized by vortex and plated onto McConkey agar plates, ESBL selective agar plates (ChromID ESBL) and blood agar plates (nonselective trypticase soy agar supplemented with 5% defibrinated human blood, hemin 5 mg/ml, and menadione 0.05 mg/ml) and incubated at 37°C for 24-48 hours in aerobic (McConkey and ChromID ESBL) and anaerobic (90% N_2, 5% H_2, 5% CO_2) conditions (blood agar plates).

Molecular methods

ESBL genotype analysis was performed using polymerase chain reaction (PCR) and in some cases sequencing. From pure cultures, approximately 10 colonies were selected from a lactose or ESBL plate and were lysed for 10 minutes at 100°C in PCR-lysis buffer (Tween 20 P9416-50 ml and Tris Ethylenediaminetetraacetic acid (EDTA) buffer (TE-buffer) 100xCons T9285-100 ml and Triton x-100 T8787 50 ml and pure water, all Sigma-Aldrich, St. Louis, Missouri) and centrifuged. The supernatant was diluted 1:10 and PCR was performed as described by Brikett et al. and $bla_{\text{CTX-M}}$ phylogroups were assigned[124]. Detection of bla_{TEM} and bla_{SHV} was performed on ESBL-positive isolates negative for $bla_{\text{CTX-M}}$ using consensus PCR followed by DNA sequencing[125]. In the oral samples 100 µl of inoculated transport medium was diluted in 100 µl of TE-buffer and frozen at -70°C. DNA was extracted using the MasterPure DNA Purification Kit (Epicentre, Madison, WI) according to the instructions of the manufacturer. PCR was then performed the same way as for the pure cultures.

The sensitivity of the entire oral PCR procedure was semi-quantitatively assessed using standardized 10-fold dilutions of CTX-M 1 producing *E. coli* (CCUG 55971) which were inoculated in the dental transport medium and used as a standard. This was done by inoculating the anaerobic transport medium with the *E. coli* strain (giving a 1/55 dilution) and then diluting 100 µl of this medium in 100 µl of TE buffer. This mixture was frozen and 150 µl (3/4) was used in the DNA extraction process which resulted in 35 µl of DNA concentrate. From this concentrate 5 µl (1/7) was used for each PCR run. The sensitivity of the PCR was 3-30 molecules per run assuming one copy of the CTX-M gene per *E. coli* (CCUG 55971). Each PCR run would include approximately ¾*1/55*1/7=1/513 of the original sample, and therefore approximately 1600-16000 CTX-M molecules would be needed in the total sample for successful detection.

Sub-typing of faecal ESBL-producing *E. coli* isolates in the oral carriage study was performed by ten-loci multiple-locus variable-number tandem-repeat analysis (MLVA) as described earlier[126,127].

Data on antimicrobial use

In Norway antibiotics are available on prescription only. The Norwegian Prescription Database collects data about all antimicrobials dispensed from Norwegian pharmacies[98]. Date, type and amount of antibiotic dispensed to all participants during the past five years were obtained from this registry. Information about antibiotic use during hospitalization was obtained from medical records.

Anamnestic data on risk factors and lifestyle

For patients included before April 2011 a structured interview was performed by a trained investigator to collect anamnestic data. The interview was done by telephone or in-person for community-based and hospitalized patients, respectively and three different investigators performed most of the interviews. The questionnaire, presented in appendix I, was sent to the participants in advance. It included detailed questions regarding the infection for which they were included in the study, health condition (Charlson Comorbidity Index[128]), contact with the health care system in Norway and abroad (time and duration during the past 5 years), previous UTIs, previous ESBL, antibiotic use, adherence to antibiotic prescriptions, prostate disease, use of a urinary catheter during the past year, oral and digestive health problems, international travel or residency lasting ≥24 hours during the past five years (time since returning home, duration of stay and country), profession, personal hygiene, household members, pets, eating habits (meals per week of different foods and meals outside home), and recreational swimming during the past year (location, number of times and submergence of head). The questionnaire was evaluated several times during the study period and underwent minor changes.

For patients included after April 2011 a shortened version of the questionnaire was used and filled in by the patients themselves (appendix II). This questionnaire included questions regarding the infection for which they were included in the study, previous UTIs, contact with the health care system in Norway, international travel, eating habits and recreational swimming.

Laboratory- and medical records data

Information on previous infections with ESBL-producing bacteria was retrieved from our laboratory`s computer systems. Medical records from hospitals and general practitioners were assessed to obtain information on antimicrobial use in hospitalized patients and for other clarifications when necessary.

Statistical analysis

The same software, univariate methods and multivariate logistic regression were used in paper I and III. No statistical analysis was performed in paper II.

The statistical analyses were conducted using SPSS statistics software, version 19.0 (IBM SPSS, Chicago, IL). Univariate analyses were performed using logistic regression, Pearson chi square, Fisher's exact test, Student's t-test or the Mann-Whitney U-test as appropriate. The association between variables and outcome was quantified by OR with 95% CI. Variables with a $p<0.15$ were considered candidates for a multivariate model. A manual backward stepwise elimination procedure using multivariate logistic regression was performed to identify independent risk factors or factors associated with treatment outcome. Multivariate analyses were preceded by estimation of correlation between risk factors and followed by testing of all initial variables added to the final model. All p-values were two-tailed, and a p-value of <0.05 was considered significant.

In paper I evaluation of the predictive accuracy of the models was assessed by calibration and discrimination. Calibration was evaluated by the Hosmer and Lemeshow goodness-of-fit test. A statistically non-significant Hosmer and Lemeshow result ($p>0.05$) suggests that the model predicts accurately on average. Discrimination was evaluated by analysis of the area under the Receiver operating characteristic (ROC) curve. We defined acceptable discriminatory capability as an area under the ROC curve greater than 0.7[129].

In paper III two different treatment outcome measures were evaluated. Cohens kappa was used to compare these.

Main results

Paper I – The case-control study

In this study we found several associations between CA-UTI caused by ESBL-producing *E. coli* and *K. pneumoniae* and lifestyle (Table 4). The strongest association was with previous travel to areas with a high prevalence of ESBL, with a stronger association for more recent travel. Recent use of fluoroquinolones and beta-lactams, diabetes mellitus and recreational swimming in freshwater were also associated with these infections. A higher age and eating fish frequently were negatively associated with ESBL-positive infections.

		Adjusted		
Variable	**Level**	**OR**	**95% CI**	**P**
Travelling to Asia, Middle East or Africa[a]				
- During the past 6 weeks	yes/no	21	4.5-97	<0.001
- Between the previous 6 weeks to 24 months	yes/no	2.3	1.2-4.4	0.017
Use of fluoroquinolones the past 90 days	yes/no	16	3.2-80	<0.001
Use of β-lactams except mecillinam in the past 90 days	yes/no	5.0	2.1-12	<0.001
Diabetes mellitus	yes/no	3.2	1.0-11	0.051
Recreational freshwater swim past year	yes/no	2.1	1.0-4.3	0.040
Age	5 year increase	0.89	0.82-0.97	0.014
Number of fish meals per week	1 meal increase	0.68	0.51-0.90	0.008

Table 4.
Independent risk factors of ESBL-positive community acquired urinary tract infection identified using multivariate logistic regression analysis.

Paper II – The oral carriage study

Oral colonization with ESBL-E or PCR-based detection of $bla_{\text{CTX-M}}$ was not observed within the frames of this study. Thus, the study turned out with negative results. The participants all had faecal samples with CTX-M producing *E. coli* but CTX-M genes or *E. coli* were not identified in the oral samples neither by culture nor by molecular methods. However, we identified an ESBL gene. This was in a *Rhanella aquatilis* which harboured a RHAN-1/2 gene. This strain or gene was not identified in the faecal samples of the patient and this finding probably represents a transient bacteria originating from water.

Paper III – The mecillinam study

In this study we found that mecillinam had reduced efficacy against ESBL-producing *E. coli* (Figure 14).

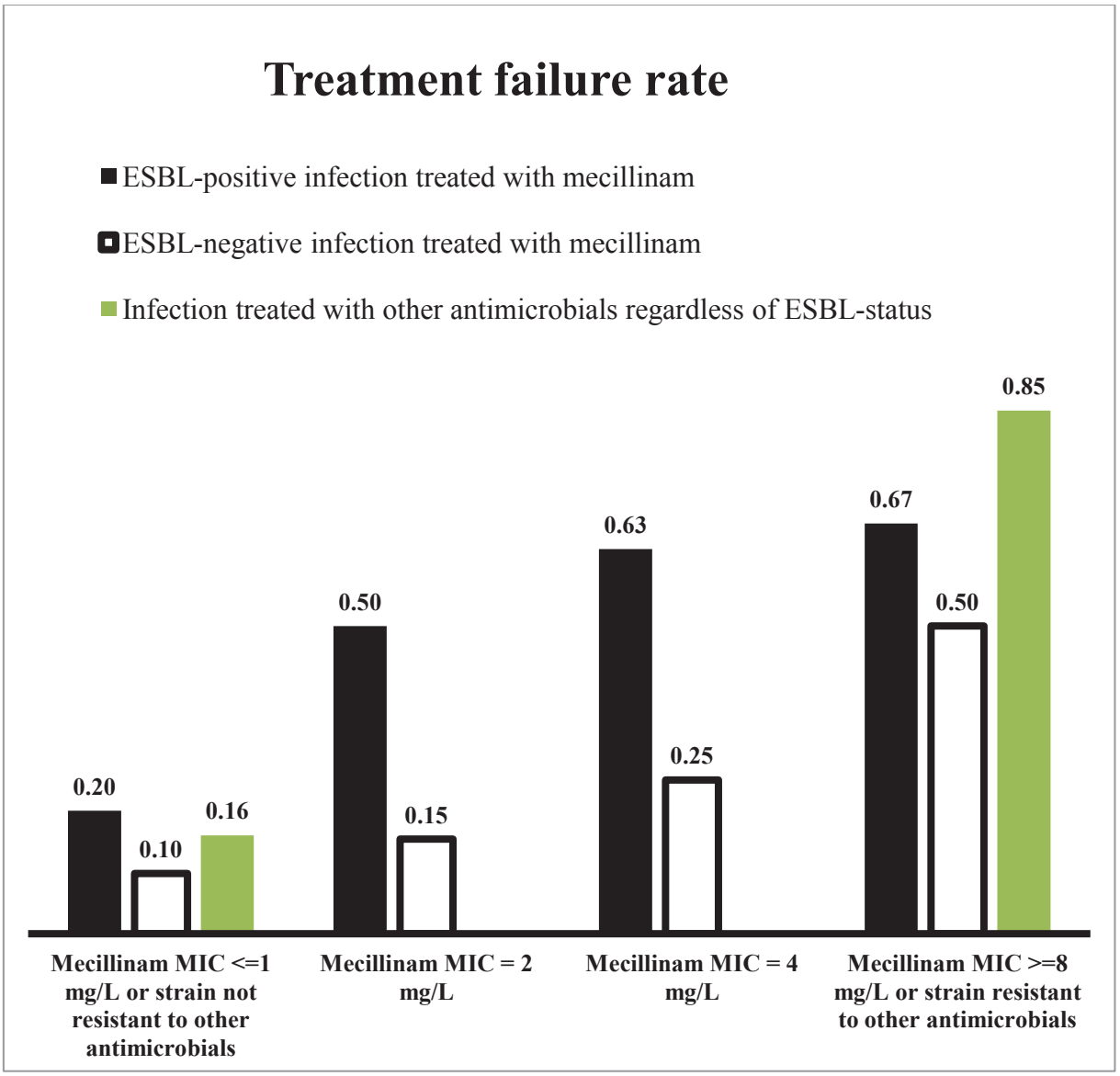


Figure 14.
Treatment failure rate in community acquired urinary tract infections caused by *E. coli*.
Black and white bars represent ESBL-producing and -non-producing strains treated with mecillinam and grouped by mecillinam mean inhibitory concentration.
Green bars represent infections treated with other antimicrobials grouped by the strain`s resistance against the treatment agent (not-resistant to the left hand side and resistant to the right hand side of the figure).

We also found an association between increased mecillinam MIC and mecillinam treatment failure that was independent of the ESBL-status of the strain. Furthermore, we found that for

other antimicrobials, the *in vitro* resistance strongly predicted treatment failure (Figure 14, green bars).The ESBL-producing and –non-producing strains had a higher frequency of *in vitro* resistance to other commonly used antibiotics than mecillinam, leading to frequent therapy failures independent of the antimicrobial chosen. Therefore, the frequency of treatment failure in the mecillinam treatment group was lower than in the non-mecillinam treatment group regardless of the ESBL-status of the patients (Figure 15).

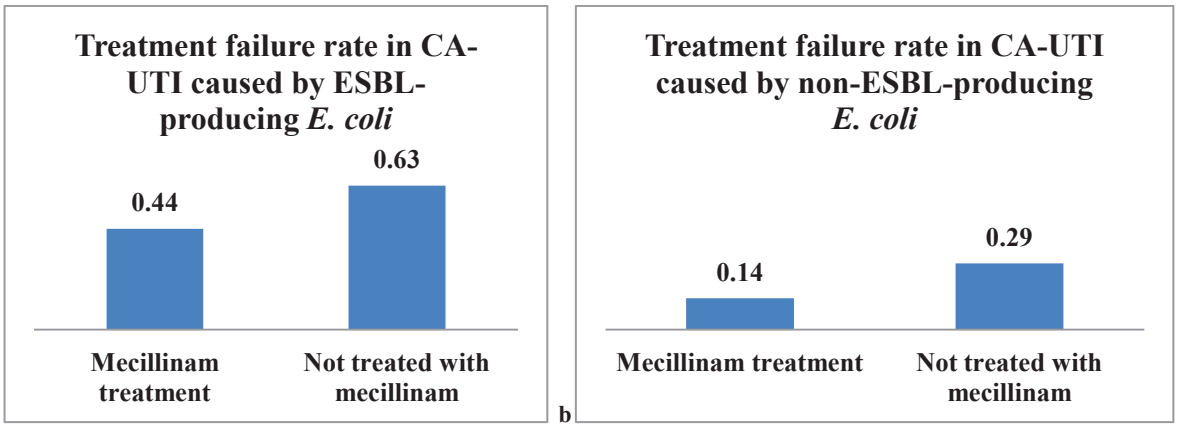


Figure 15.
The treatment failure rate in community acquired urinary tract infection caused by *E. coli* was higher in patients with an ESBL-producing strain (a) than in patients with a non-ESBL-producing strain (b). Patients not receiving mecillinam (i.e., treated with other antimicrobials), had the highest rate of treatment failure. The difference was not significant in the ESBL-positive group (p=0.12) while it was significant in the ESBL-negative group (p=0.004).

The mecillinam treatment failure rate among non-ESBL producing ampicillin resistant *E. coli* was 8/42 (19%) while in non-ESBL ampicillin sensitive *E. coli* the corresponding rate was 8/75 (11%). This difference is not significant (p=0.26) (Figure 16). However, this is interesting because it might suggest that TEM-1, the most common cause of ampicillin resistance, may also hydrolyze mecillinam.

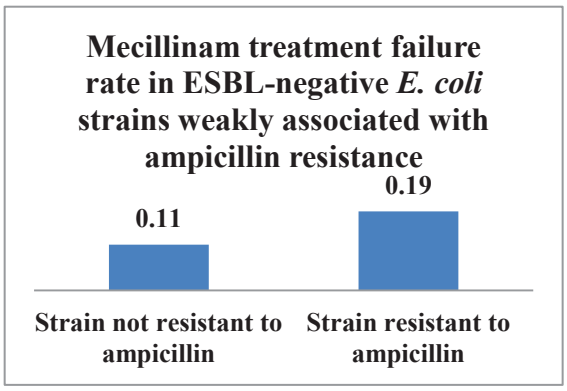


Figure 16.
The mecillinam treatment failure rate in ESBL-negative strains of *E. coli* was lower in strains not resistant to ampicillin than in ampicillin resistant strains, but the difference was not significant (p=0.26)

Discussion

Discussion of methods

The methods used are discussed in the papers, but some key points will be elaborated here.

Data collection

The ESCAPE project was planned with the aim to elucidate important questions regarding the epidemiology of ESBL. We therefore collected information not only about the bacterial isolates from the original UTI, but also from a wide range of other sources. After agreeing to participate the patients and controls were interviewed and data from the Norwegian Prescription Database and from medical records were obtained. Additionally, we included in the project all volunteering household members of patients including pets, and collected faecal samples from all participants including serial samples from patients with ESBL. Moreover, simultaneous environmental sampling (water and sewage) was performed. To increase the strength of the study an additional batch of patients was included in June 2012. These patients were not interviewed and did not submit a faecal sample, but filled in a shortened questionnaire and prescription database- and medical records data were obtained.

Paper I – The case-control study

This study design compares participants with a condition to participants without the condition. It is a classical observational study design which is particularly suited in rare diseases and in diseases where little is known about the risk factors like CA-UTI caused by ESBL-E in Norway[130]. Accordingly, it was natural choice for the aims of our study. A reasonable proxy for acquisition of these bacteria is CA-UTI because this is often the first chance to detect the ESBL-producing bacteria. Thus, the risk factors identified may also reflect risk factors for acquisition of ESBL-E in Norway in general.

We chose patients with CA-UTI without ESBL as the control group. Another possible control group could be the normal population. The risk factors we would identify with that control group would be for both CA-UTI and CA-UTI caused by ESBL-E and therefore we would in any case also need a second control group with patients with CA-UTI without ESBL to single out the risk factors for ESBL-E UTI alone. This case-control-control design has been used in other studies and would, as discussed in paper I, provide the best estimation of OR with regard to use of antibiotics[131-133]. However, use of antibiotics is maybe the best described risk factors for infections caused by ESBL-E. Therefore a case-control study design with only

one control group was chosen. Another reason for this was to include the highest possible number of cases within our restricted resources. This enabled us to study less frequent risk factors in greater detail.

Low response rates in case-control studies are important sources of selection bias[134]. Therefore, a number of measures were applied to increase the response rate in the study. The questionnaire were kept as short as possible and, for convenience, the participants were interviewed instead of filling in the questionnaires themselves. Furthermore, non-responders were contacted twice. To further increase the overall response rate participants were incentivized by participation in a $1.700 travel gift card draw. This may have introduced its own bias because this incentive may work best among possible participants with a lower socioeconomic class as well as those who enjoy travelling, but we prioritized a high overall participation over the probable small bias introduced by the gift card draw. For ethical and practical reasons the participants were informed about their ESBL-status when they were invited to participate. Thus, participants with an "interesting" UTI caused by an ESBL-producing strain may have been influenced by this information when they decided whether to participate or not, thereby introducing another selection bias. The knowledge about the ESBL-status may also have introduced a recall bias to the study as patients with ESBL-E may recall the risk factors investigated differently from patients without ESBL-E.

It was not feasible to hide the information about ESBL-status from the interviewers and this may have resulted in differential misclassification of exposures. To minimize the latter the questionnaires were sent to the participants in advance and the interviewers underwent interview training.

Another methodological challenge in paper I is that the questionnaire used included a relatively high number of questions and with the 5% CI used in the study it is possible that variables have been identified as being associated with ESBL-UTI by chance. This is probably most relevant for previous unidentified associations with a p-value close to 0.05 (i.e., recreational freshwater use and fish consumption), and further studies are needed to clarify these findings.

Regarding study size: The number of patients included in the study was exactly 100. This was more than the 70 initially planned for. The decision of increasing the number of participants was done early in the study period because a higher number was needed for studying the duration of faecal carriage of ESBL-producing bacteria which was another goal for the ESCAPE project. Moreover, the higher number of participants allowed for a more precise estimation of the strength of the associations than initially planned for.

The correct classification of patients into cases or controls is vital in case-control studies. The laboratory methods used in paper I with the combination of VITEK based screening for ESBL-E, phenotypic confirmation using E-test and genotypic verification using PCR makes the chance of false cases or controls low. However, when we collected faecal samples from the participants and screened the samples for ESBL-E six controls were faecal ESBL-carriers. This finding was not unsuspected since an ESBL-E in the gut not necessarily causes a UTI. The number was low and either reclassification or exclusion of these controls did not affect the results from the study significantly, and they were therefore included according to the study protocol.

Another possible source of selection bias must be mentioned in this study because only patients who had their urine sent to our laboratories for culture could be included. Norwegian guidelines, which are partly adhered to by the general practitioners, stipulate that culturing is unnecessary in uncomplicated UTI[135]. Thus, a high number of uncomplicated UTIs were not considered for inclusion and the study population were probably enriched in patients with complicating factors like high age, suspicion of pyelonephritis, urinary catheters, urinary abnormalities and recurring infections. The study results may only be extrapolated to the group of patients who had urines submitted for culturing.

Paper II – The oral carriage study

In this sub-study, we aimed to investigate possible oral carriage of ESBL-E in known faecal carriers of ESBL-E. Very little was known about this, and in this regard the study can be considered to be a pilot study. A methodological weakness is that faecal ESBL carriage status was based on the serial faecal samples collected instead of a faecal sample collected at the time of oral sampling. Consequently, a time interval passed between the last confirmed faecal sample and the oral sampling and also between the oral sampling and the next faecal sampling. This led to an uncertainty about the true faecal carriage status of the participants at the exact time of oral sampling, weakening the conclusions that could be drawn from the results.

However, there are reasons to believe that the results obtained from each oral or faecal sample reflects the true ESBL-status because results from culture- and PCR-based methods were concordant in every sample. The sensitivity of the DNA extraction- and PCR procedures used on the oral samples were estimated to 3-30 gene copies per PCR run, which is considered acceptable.

Paper III – The mecillinam study

The overall participation rate in this study was lower than the case-control study. This was partly because approximately 1/3 of the patients did not receive an antimicrobial at the day of their urinary sample. Furthermore, the participation rate among patients invited in June 2012 was lower than for the previously invited patients. Bias related to the participation rate has been discussed in paper III.

A repeat prescription of an antimicrobial adequate to treat UTI within 14 days of initiation of treatment was considered treatment failure in this study. Evaluation of treatment outcome also by urinary culturing would have given additional information, but this was not a part of the study and the clinical treatment outcome was evaluated through interview. The interviewers were blinded to the treatment agent and the treatment outcome derived from the prescription database. The clinical outcome was congruent with the outcome based on prescription registry data in 87% of the evaluable cases, but 27% of the patients did not give adequate information during the interview to evaluate the treatment outcome. This was probably due to the time lapse between the infection and the interview as interviews were not always performed immediately after the infection.

An important aspect of this study was that we, in addition to information on mecillinam treatment, also collected information about participants treated with other antimicrobials. It was therefore possible to extract information not only regarding how ESBL-production affected the efficacy of mecillinam treatment, but also regarding how ESBL-production affected treatment with these alternative antimicrobials. The ESBL itself would have no activity against these non-beta-lactam antimicrobials, but other traits of these strains, for instance ESBL-associated virulence factors, might still cause adverse treatment outcomes. Thus, information on how such virulence factors and other traits possibly associated with ESBL-production affected treatment outcome could be extracted and the clinical effect of ESBL-production against mecillinam could be deduced and separated from other effects related to the bacteria producing ESBL.

Discussion of results

Most of the results are discussed in the respective papers, but some interesting findings need elaboration.

Paper I – The case-control study

Time of infection after travel

Our data showed that the OR for ESBL-positive infection was significantly elevated in participants who had travelled to high risk areas of intestinal colonization with ESBL like Asia, Middle East and Africa[61]. This has been discussed in the paper and is consistent with other studies also published after paper I[61-63,136]. The time course between travel and infection can be explored in more detail. In Figure 17 the participants have been divided into groups according to time since most recent travel to these areas.

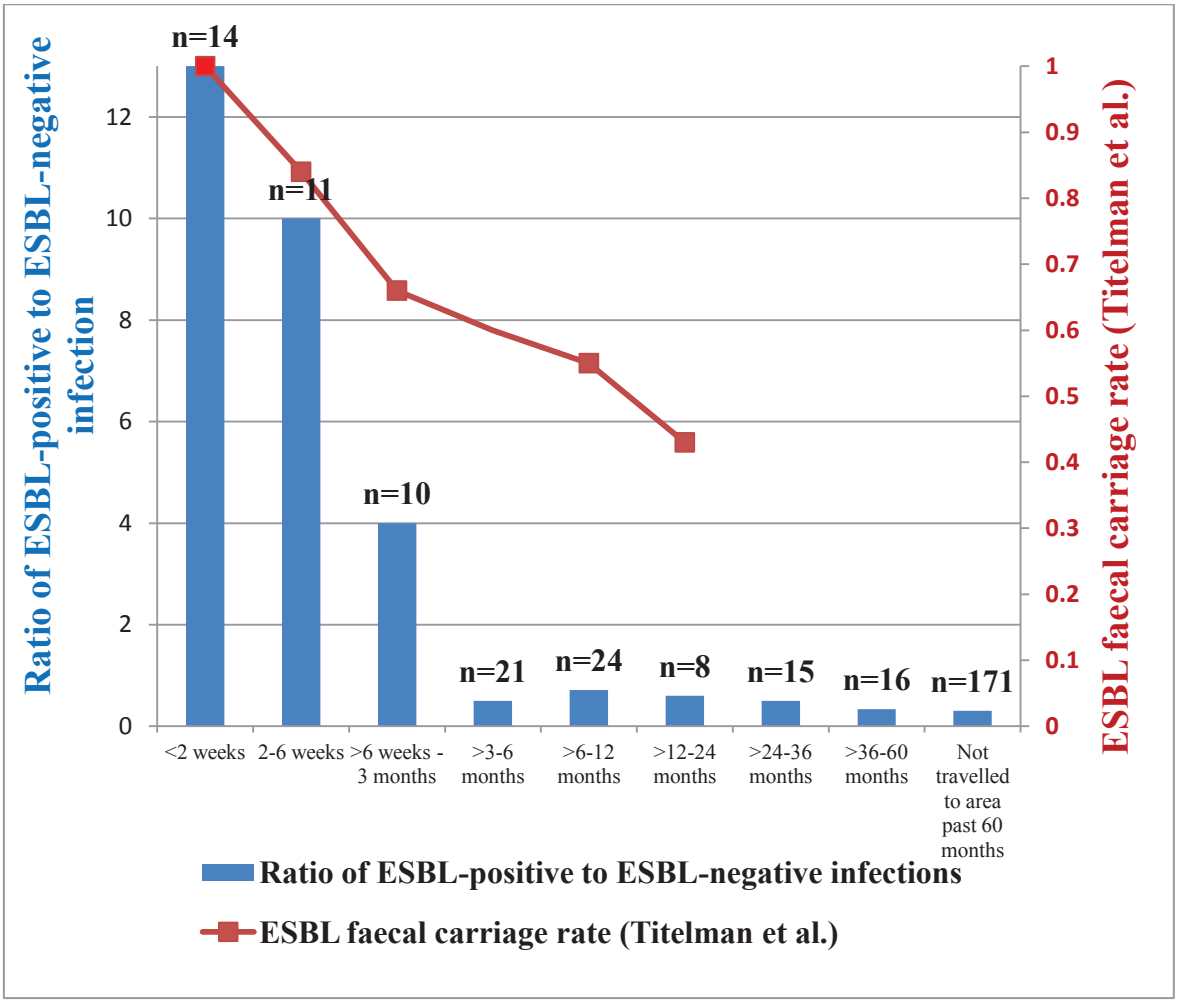


Figure 17.
The study participants divided into groups according to time since most recent travel to Asia, Middle East or Africa (Blue bars – left axis). The case/control ratio is high in participants who have recently travelled to these areas. Numbers of participants in each group is stated above the bars.
The faecal carriage rate of ESBL-E after infection with ESBL-E as it was observed in a study by Titelmat et al (Red line – right axis)[76]. First point (bright red) is extrapolated to 1.0 (i.e., a carriage rate of 100%).

The figure indicates that an elevated risk of ESBL-positive CA-UTI lasts for only about three months. A recent study by Titelman et al. in Sweden found that the faecal carriage rate after infections caused by ESBL-E were 84%, 66%, 55% and 43% after one, three, six and twelve months, respectively (Figure 17)[76]. The high rates of faecal carriage after six and twelve months contrast our finding that the risk of ESBL-positive infection was reduced to baseline already three months after returning home. This suggests that one cannot assume that the risk of infection due to ESBL-E equals the risk of ESBL-E. The reason for this is unclear.

Freshwater swimming was found to be associated with ESBL-positive infection. Colonization of the gastrointestinal or urinary tract by ESBL-producing bacteria from the water is the probable cause of this *if* a causal relationship actually exists. In one study of an *E. coli* O157 outbreak among recreational swimmers in a lake the authors found that *E. coli* O157 in the suspected water had the same restriction fragment length patterns as the patient isolates and thus established a strong epidemiological link[137]. Environmental samples collected as part of the ESCAPE study identified at least one public freshwater beach in our area where ESBL-producing *E. coli* was regularly identified. Probably several such beaches exist in our area and they may represent a source of ESBL colonization and infection.

Participation rate

The overall participation rate was 47% and was higher among cases (58%) than among controls (43%). The relatively low participation rate must be considered in relation to our request for faecal samples over a long period of time. Also the number and kind of questions in the questionnaire may have had effect. The patients were only contacted twice, and only by mail. This approach may also have contributed to a reduction in participation.

A majority of the patients refusing to participate did so passively by not returning the consent form. We have little information about these patients except gender and age. The gender distribution was similar among participants and non-participants. Non-participating cases and controls were 1.6 and 4.5 years younger than participating cases and controls, respectively. This difference was significant for the control group ($p=0.02$).

Interestingly, the lower participation rate among controls compared to cases was only observed in the age groups <60years and >90years (Figure 18).

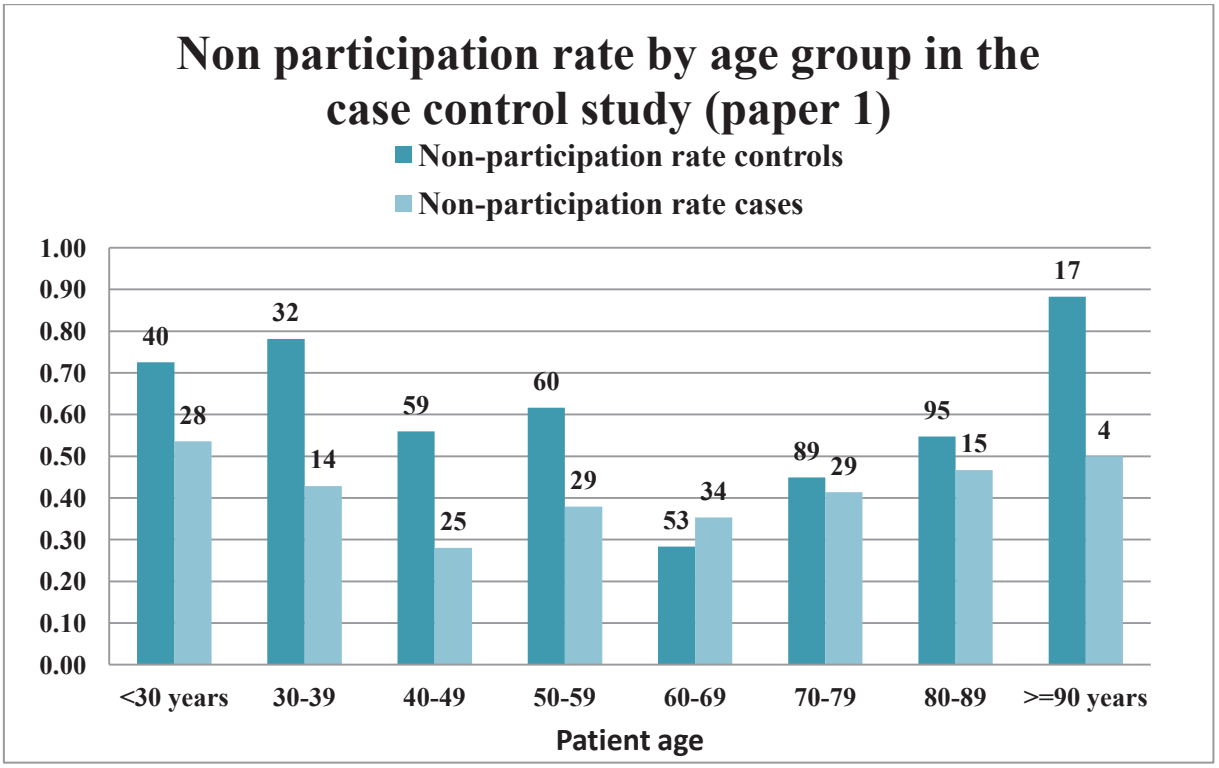
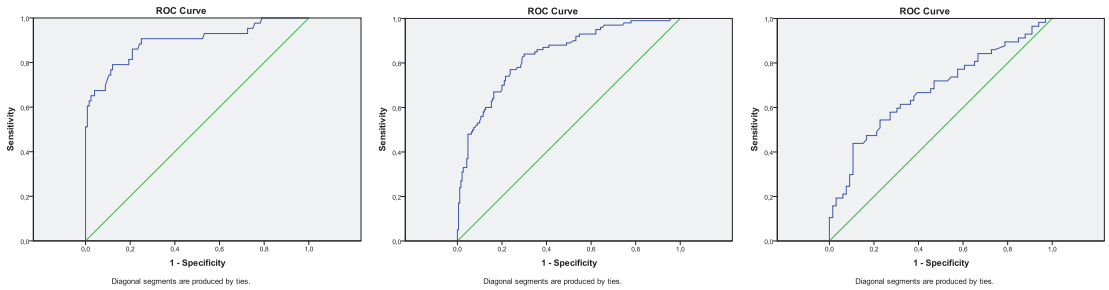


Figure 18.
The non-participation rate in the case-control study was similar in patients and controls only for the "middle" age group (60-89 years), while among younger and older patients the non-participation rate among controls was higher than for cases. Digits on top of bars are number of participants.

Since we have scarce information about the non-participants, it is difficult to determine how the variation in participation of controls compared to cases has influenced the study outcome. Rerunning the final model in the groups with the best participation rate in the control group 60-89 years (n=167), give similar results as the final model presented in paper I, and the discriminatory ability of the model for these patients was very good and even better than for the whole population (Figure 19) (area under the ROC curve (AUC) =0.895, CI 0.83-0.96). Among the other participants with ages <60 or >89 years, n=123) the value for the final model was low (AUC 0.68, CI 0.58-0.77).

The better model discrimination may indicate that the non-participation among controls with "extreme" ages did affect the study outcome, but it may just as well indicate that the risk factors identified apply more to the "middle" age groups and that the different groups have different sets of risk factors.



60-89 years (AUC=0.89) | All patients (AUC=0.83) | <60 and >89 years (AUC=0.68)

Figure 19.
Receiver operating characteristic curves showing that the discriminatory ability of the final model in the case-control study was best among patients aged 60-89 years which is also the age group with a similar non-participation rate among patients and controls.

Paper II – The oral carriage study

All the participants had a faecal CTX-M producing *E. coli*. No *E. coli* or CTX-M were identified in the oral samples. The result is weakened by the low number of participants (n=17), but since no positive result was obtained indicates that faecal occurrence of ESBL is not as a rule associated with oral carriage. However, hospitalized patients and patients under treatment with certain antimicrobials have been shown to be at higher risk for oropharyngeal carriage of Enterobacteriaceae[138,139]. In one study from the Netherlands Filius et al. found the prevalence of oropharyngeal carriage of Enterobacteriaceae to be 1.1%, 3.4%, 12.4%, 19.4% and 20.3% in patients at admission, during stay, at discharge and 1 and 3 months after discharge from hospital, respectively[138]. This situational increased prevalence of carriage may be important for transmission of these bacteria that can carry resistance genes, in particular in hospitals. We do not know of studies addressing carriage of ESBL-E in the oral cavity prior to our preliminary study. This area might clearly be an area of future research.

Paper III – The mecillinam study

The main result presented was that mecillinam has a moderately reduced efficacy against ESBL-producing *E. coli.*

The inclusion in this study of a non-mecillinam treatment group combined with information on antimicrobial resistance of the bacteria made it possible to compare the antimicrobial resistance-corrected treatment outcome between patients treated with mecillinam and with other antibiotics and in relation to ESBL-producing strains and non-ESBL-producing strains. Thus, any impact of ESBL-production on mecillinam could be isolated. In the paper the mecillinam treatment group and non-mecillinam treatment group were analysed separately. Yet, the whole material may be analysed together using multivariable logistic regression including the combined variables "ESBL-positive UTI treated with mecillinam" and "ESBL-positive UTI treated with other antimicrobials" in the initial multivariable model. In this case the latter variable was eliminated from the final model as non-significant whilst the former would be included in the final model (OR=2.6, 95% CI: 1.2-5.8, p=0.02). An OR >1 implied increased risk of treatment failure, i.e., the mecillinam efficacy was significantly reduced specifically in ESBL-producing strains, while other antimicrobials did not have a significantly reduced efficacy in these strains apart from what would be expected from their antimicrobial resistance.

We found a reduced clinical efficacy of mecillinam against ESBL producing *E. coli* that was partly related to the mecillinam MIC of the strain. This indicates that a higher dose of mecillinam may have a potential to enhance its efficacy. A recent study by Jansåker et al. partly supports this and reports a bacteriological cure rate of 80% in 30 patients with UTI caused by ESBL-E and treated with pivmecillinam 400 mg three times a day (TID)[100]. The study also included nine patients receiving 200 mg TID who experienced the same treatment outcome (78% bacteriological cure rate), but this number was too low for any meaningful comparison with our data. Altogether Jansåker found clinical effect in 16 of 19 (84%) patients. Twelve patients did not report sufficiently on clinical outcomes and this included many of the older patients and nursing home residents. Therefore, comparison to our study where 14/31 (55%) of the evaluable patients did not experience a clinical relapse is difficult.

The high frequency of co-resistance found in the ESBL-producing strains also significantly reduced the efficacy of other antimicrobials used in empirical therapy in UTI. Even at the low dose (200 mg TID) probably dispensed to our patients the mecillinam group came out better than the non-mecillinam group, regardless of ESBL-production, indicating that mecillinam is a useful alternative as empirical therapy in all settings regardless of the ESBL prevalence (Figure 15).

Finally, it has been reported that TEM-1 may hydrolyse mecillinam[99,140]. In our study TEM-1 genotyping was not performed, but TEM-1 is the most common cause of ampicillin

resistance in *E. coli*[9]. We did not find a significant difference in the mecillinam treatment failure rate between ampicillin resistant and non-resistant strains of non-ESBL-producing *E. coli* (p=0.26). However, to rule out that TEM-1 has a weak anti-mecillinam activity a larger study with definitive identification of TEM-1 is needed.

Future perspectives

We have drawn some conclusions from our work, but many aspects regarding the spread of ESBL-E, and in particular CTX-M producing *E. coli,* remain to be investigated.

Environment and food

One area of ongoing dispersion of ESBL is into the environment through disposal of human waste (sewage) and animal waste (manure). The clinical effect of this is poorly described, and the association we found between recreational freshwater swimming and ESBL-UTI is interesting in this respect. This finding remains to be confirmed in other studies. It is, however, likely that a wide dispersion of ESBL in the environment will affect human health either directly, as suggested in paper I, or indirectly through food or other sources.

Another area of ongoing dispersal of ESBL is into the food production chain in Norway where $ESBL_M$ is found in retail chicken on a regular basis and in other countries where also $ESBL_A$ is found in such products[19,66]. This is a cause of great concern and this should, from a clinician's point of view, be prevented. Unfortunately, effective changes in food production are probably both expensive and difficult to implement.

ESBL-genes represent unwanted biological substances and their dispersion into the environment and into food may be considered a "new" type of pollution. On the other hand, preventing such spread may also be considered a new area of possible intervention against the increasing spread of antimicrobial resistant bacteria in the population.

Antibiotic use in humans

Human antibiotic consumption is an important driver of the spread of ESBL and other resistance mechanisms. As ESBL-producing bacteria often harbour multiple resistance mechanisms, a general reduction of human antibiotic consumption, and in particular of cephalosporins and fluoroquinolones, is probably needed to have an impact on the further spread. Increased use of narrow spectrum antibiotics instead of broader acting ones may also have an impact. Our study and other studies have shown that mecillinam in adequate doses may substitute other antimicrobials used in the treatment of CA-UTI regardless of the ESBL-prevalence. Another approach worth mentioning in this regard is the possibility to prescribe

anti-inflammatory drugs instead of antibiotics to enable the infection to resolve itself. An ongoing Norwegian study is investigating this (ClinicalTrials.gov Identifier: NCT01488955).

Data from archived soil samples indicate that plasmid associated penicillinases like TEM-1 became much more common after the introduction of penicillins[141]. In the late 1970s TEM-1 was the most common cause of ampicillin resistance in the UK and in UTIs the prevalence was approximately 20%[56]. Today, 30 years later, the prevalence of ampicillin resistance in clinical *E. coli* isolates in Europe is above 55%[9]. An unfavourable combination of bacterial strains, plasmids and genes in a fruitful environment has been the key to a long term increase of TEM-1 in Europe. This is an ominous sign demonstrating that Europe is not immune to antimicrobial resistance and consequently may experience the same development regarding CTX-M as has already happened in South Asia, if no action is taken.

Encouragingly, several European countries have recently initiated an ambitious research programme with an overall aim to counteract antimicrobial resistance and to initiate the development of new innovative antimicrobial strategies. One might hope that increased research and awareness may reduce animal and human antibiotic use and thereby limit the spread of antimicrobial resistance genes (http://www.jpiamr.eu/). This thesis suggests that the society should aim to reduce the concentration and spread of resistance genes wherever they are found. Antibiotics are a prerequisite to the practice of modern medicine; humanity cannot afford to lose the anti-infective effect of these drugs.

Conclusions

The results of the studies enabled us to draw conclusions on the questions initially asked.

Paper I – The case-control study

We found that the risk of ESBL-positive CA-UTI was influenced by foreign travel, eating less fish and recreational freshwater use and also to the known risk factors antibiotic consumption and diabetes mellitus.

Paper II – The oral carriage study

We did not find that supragingival plaques were colonized by ESBL-producing bacteria in intestinal carriers of such strains.

Paper III – The mecillinam study

We found that mecillinam had a useful clinical effect in the treatment of CA-UTI caused by ESBL-producing *E. coli*, however lower than for non-ESBL-producing strains. Due to a high prevalence of multiple resistances in ESBL-producing strains, mecillinam seems to be an acceptable empirical alternative also in areas with a high ESBL-prevalence.

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Appendix I

4. OBS: DETTE SPØRRESKJEMAET FYLLES UT PÅ TELEFON OG ER VEDLAGT SOM ET EKSEMPEL SLIK AT DU SKAL VITE HVILKE SPØRSMÅL VI VIL STILLE

– DU SKAL IKKE FYLLE UT/SENDE INN DETTE!

Spørreskjema ESBL bærerstudien

Navn på den som fyller ut spørreskjemaet: _____

Bekreftelse på at informasjon om studien er gitt (Signatur): _____

Dato for utfylling: _____

Dato for overføring til databasen: _____

Studienummer: _____

Spørreskjema gjelder en:

Sett kryss	
Pasient	
Kontroll	
Husstandsmedlem	

Høyde og vekt:

Fyll inn	
Høyde	
Vekt	

Resultat av urinprøve (hvis aktuelt)

Sett kryss/fyll inn		
	Ja	***Nei***
E. coli		
K. pneumonia		
ESBL positiv prøve		
Antall dager fra sykdomsdebut prøven ble tatt		
Dato for urinprøve		

Tidsforløp for urinveisinfeksjonen:

Hvis dag 0 var da du første gang merket symptomer på UVI. På hvilken dag:

Skriv inn nr for hvilken dag	
	Dag nummer
Fikk du tatt urinprøven som gjorde at du ble spurt om å v med i studien	
Begynte du på første antibiotikakur	
Antibiotikakur nr 2	
Antibiotikakur nr 3	
Innlagt/utskrevet fra sykehus	*/*
Hvilken dag ble du frisk?	
Andre merknader til forløpet av infeksjonen:	

1. YRKE

Hvilket yrke har du nå? (velg det som passer best)

Sett kryss	
	Annet
	Landbruk
	Helse/barn/omsorg
	Renovasjon/rengjøring/hotell
	Kontor
	Undervisning/forskning
	Butikk,
	Næringsmiddelindustri/restaurant
	Ttransport
	Pensjonist

Kort beskrivelse av yrket: _____

Hva har du jobbet mest med de siste 3 årene? (velg det som passer best)

Sett kryss	
	Annet
	Landbruk
	Helse/barn/omsorg
	Renovasjon/rengjøring/hotell
	Kontor
	Undervisning/forskning
	Butikk,
	Næringsmiddelindustri/restaurant
	Ttransport
	Pensjonist

Kort beskrivelse av yrket: _____

Jeg har hatt nærkontakt med mennesker/dyr i mitt yrke i perioder på >6 måneder i løpet av de siste 5 år:

Sett kryss		
	Ja	***Nei***
Mennesker		
Dyr		

2. Noen Ja/Nei spørsmål:

Jeg har vært innlagt på sykehus eller har vært på sykehjem siste 5 år (regn bare med innleggelser som varte mer enn 24 timer)

Sett kryss		
	Ja	***Nei***
Sykehus		
Sykehjem		

Jeg har vært i utlandet siste 5 år (regn bare med innleggelser/opphold som varte mer enn 24 timer)

Sett kryss		
	Ja	***Nei***
Jeg har vært i utlandet siste 5 år		
Jeg har vært på sykehus i utlandet siste år		

Antibiotikabruk siste 5 år

Sett kryss eller skriv inn			
	Ja	***Nei***	***Vet ikke***
Jeg har brukt antibiotika i Norge siste 5 år			
Jeg har brukt antibiotika i utlandet siste 5 år			
Hvis Ja: *Hvilke(t) land?* *Hva slags antibiotika (evt for hva fikk du antibiotika)?* *Når var dette?*			
Jeg har brukt antibiotika siste 5 år som ikke var skrevet ut til meg			
Hvis Ja: *Hva slags antibiotika (evt for hva tok du antibiotika)?*			

Bytte av yrke siste 3 år

Sett kryss		
	Ja	***Nei***
Jeg har byttet yrke de siste 3 årene		

3. ANTIBIOTIKA

Antibiotikabruk i utlandet siste 5 år.
(Dette spørsmålet gjelder ikke antibiotika gitt på sykehus eller sykehjem).

Skriv inn hvor lenge siden forsøkspersonen var i utlandet under Periode (eksempel "5 mnd" betyr 5 måneder siden)

Periode	***Område/land***	***Type antibiotika(navn om mulig)***

Antibiotikabruk i Norge siste 5 år – egne resepter.

Periode	***Type antibiotika(navn om mulig)***

Avbrutte antibiotikakurer – sett kryss

	Ja	**Nei**
Har du brukt opp alle antibiotikakurer siste 5 år		
Jeg har avbrutt/ikke brukt en eller flere antibiotikakurer siste 5 år		
Hvis avbrutte/ikke brukte kurer: Når var det?		

Antibiotikabruk i Norge siste 5 år – preparater som egentlig var skrevet ut til andre.

Periode	***Type antibiotika(navn om mulig)***

4. Innleggelser i Norge (bare hvis har vært innlagt på sykehus siste 5 år)

Innleggelser som har vart >24 timer på sykehus eller sykehjem siste 5 år – i Norge:

Forklaring:
Tidsperioden oppgis i antallet mnd. før urinprøven som førte til inkludering i studien ble avlagt
Hvis prøven ble tatt i mottakelsen på sykehuset krysses i boksen for 0-1 mnd.

Hvis du var på intensiv avdeling eller overvåkningspost når du var på sykehus så kryss av for dette også.

Kryss av for når innleggelsene var: (flere kryss er mulig)						
Type institusjon	***Siste 0-1 mnd***	***Siste 2-3 mnd***	***Siste 3-6 mnd***	***Siste 6-12 mnd***	***Siste 1-2 år***	***Siste 2-5 år***
Sykehus						
Sykehjem						
Intensiv avdeling på sykehus						

Fikk du antibiotika på disse stedene
(sett ring rundt)

Type institusjon	***Siste 0-1 mnd (eksklusjon skriterium!)***	***Siste 2-3 mnd***	***Siste 3-6 mnd***	***Siste 6-12 mnd***	***Siste 1-2 år***	***Siste 2-5 år***
Sykehus	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*
Sykehjem	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*	*Ja/Nei/ Vet ikke*

Innleggelser på sykehus i Norge (forts.)
Hvilke sykehus var du innlagt på?

Periode	***Navn på sykehus***

5. REISE

Utenlandsreiser
Hvis du har vært i utlandet – Hvor lenge siden, når og hvor?

Fyll ut en linje for hver reise

Område/land	*Periode*	*Varighet av opphold*

Innleggelser på sykehus i utlandet
Hvis du har vært innlagt på sykehus i utlandet – Hvor lenge siden, når og hvor?

Fyll ut en linje for hver innleggelse

Område/land	*Periode*	*Varighet av opphold*

6. HYGIENE

Hvor ofte dusjer / bader du pr uke?

Sett kryss:			
3-7 ganger	*1-2 ganger*	*Ca hver 2. uke*	*Sjeldnere enn hver 2. uke*

7. BADING

Antallet ganger du har badet forskjellige steder siste år.

Sett kryss:					
Sted	*Ingen*	*1-5 ganger*	*Mer enn 5 ganger*	*Bare i Norge (se ring)*	*Jeg pleier å duk hodet når jeg*

						bader (sett ring)	
Sjøvann				*Ja*	*Nei*	*Ja*	*Nei*
Ferskvann				*Ja*	*Nei*	*Ja*	*Nei*
Basseng				*Ja*	*Nei*	*Ja*	*Nei*

8. BRIUK AV ANTIBAKTERIELLE MIDLER I HJEMMET

Bruker du regelmessig: (i den siste måneden før inklusjonsdato)

Sett kryss			
Produkt	**Ja**	**Nei**	**Vet ikke**
Colgate Total (BARE "Total"!)			
Colgate munnskyll			
Annet munnskyllevann (klorhexidin?			
Zalo antibakteriell			

9. MAT

Antall dager per uke du spiser minst 1 porsjon av forskjellige matvarer
1 porsjon er >150g kjøtt/fisk eller 1 håndstørrelse av andre matvarer
(rund oppover hvis svaret er for eksempl"3-4")

Sett kryss for antallet dager per uke								
Matsort	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Kjøtt								
Fisk								
Grønnsaker								
Brødmat/kornprodukter								
Organisk/økologisk dyrket mat								
Restaurant/storkjøkkenmat/kantine								
Middag på restaurant								

Hvordan liker du kjøttet (for eksempel biff) ditt stekt?

Sett kryss:		
Rått (lite stekt)	**Medium**	**Mye (gjennomstekt)**

10. HUSDYR

Kryss av for hvilke(t) husdyr du eventuelt har:

Sett kryss

Type dyr	*Ja*	*Nei*
Katt		
Hund		
Hest		
Annet (skriv inn)		

Antibiotikabruk blant husdyr

Sett kryss			
	Ja	*Nei*	*Vet ikke*
Et eller flere av husdyrene har brukt antibiotika siste 5 år.			

11. HUSSTANDSMEDLEMMER

Antall husstandsmedlemmer _____ (inkludert pasienten)

Spørsmålene nedenfor gjelder de andre husstandsmedlemmene – ikke pasienten

Sett kryss			
	Ja	*Nei*	*Vet ikke*
Et eller flere husstandsmedlemmer har vært innlagt på sykehus i Norge de siste 5 årene			
Et eller flere husstandsmedlemmer har vært innlagt på sykehus i utlandet de siste 5 årene			
Et eller flere av hussandsmedlemmene har brukt antibiotika siste 5 år.			

12. BESØK FRA UTLANDET

Sett kryss		
	Ja	*Nei*
Har husstanden hatt au pair i løpet av det siste året		
Hvis ja: hvor fra?		
Har husstanden hatt besøk (varighet >1 døgn) utlandet i løpet av det siste året?		
Hvis ja: hvor fra og hvor mange dager totalt?		

13. SYKDOMMER

Så vidt du kjenner til – har du noen for tiden noen av de følgende sykdommene?

Sett kryss		
	Ja	***Nei***
Astma, emfyzem, kronisk bronkitt eller KOLS		
Rheumatisk sykdom (bindevevssydkom, RA, Sjøgren osv)		
Kreft diagnostisert siste 3 år (inkluderer lymfe- og blodkreft		
Kreft med spredning (gjelder bare tumores, ikke blodkreft) diagnostisert siste 3 år		
Diabetes		
Diabetes med organskade (for eksempel nyresvikt, skade pa netthinne, fotsår osv)		
Problemer med fordøyelsen (som for eksempel kolitt, mages eller galleblærebetennelse)		
Hjerteproblemer (slik som angina, hjertesvikt eller koronarsykdom)		
HIV		
AIDS		
Nyresykdom		
Alvorlig nyresykdom		
Leversykdom		
Alvorlig leversykdom		
Hjerneslag		
Prostatasykdom (ny170210)		
Omtrentlig antall urinveisinfeksjoner siste året (inkludert den som gjorde at du ble inkludert i studien. (ny17021		

ANNET

Sett kryss			
	Ja	***Nei***	***Vet ikke***
Jeg har gjennomgått trippelkur/antibiotikakur for magesår (oftest 2-3 tabletter per dag i rundt 2 uker)			
Jeg har fått påvist tannkjøttsykdom (gingivitt, periodontitt, pyoré)			
Jeg har mye plager med tannkjøttet			
Jeg har mye problemer med hard mage/obstipasjon			
Jeg har mye problemer med løs mage			
Jeg har brukt probiotika tabletter i løpet av siste år			

(for eksempel Idoform)			
Jeg bruker melkeprodukter med probiotika regelmessig (minst 1 gang per uke). (for eksempel Youghurt, Biola o.l)			
Jeg har barn som går i barnehage eller er ofte (>5 timer per uke) i kontakt mer barn som går i barnehage.			
Navnet på den deodoranten du oftest bruker (hvis du husker):			
Navnet på legen som du oftest bruker:			
Jeg har hatt blærekateter (urinkateter) i løpet av siste måned			
Jeg har hatt urinkateter i løpet av siste år			

14. MEDISINER

Navn på medisiner som du bruker fast (dose trengs ikke)

15. Andre spørsmål

Kan vi inkludere følgende i studien?

(vi vil sende ut eget samtykke skjema til eventuelle husstandsmedlemmer som oppgis)

Sett kryss			
	Ja	***Nei***	
Husstands-medlemmer			*Skriv inn navn:*
Husdyr			*Hvilke (skriv inn navn eller type):*
Til slutt: Ønsker du å vite svar på avføringsprøvene? *(sett ring rundt)* Ja Nei			

Appendix II

Spørsmål og samtykke

Spørsmålene nedenfor gjelder året før prøven ble tatt

- altså tidsperioden fra xx til xx

Hvor mange dager per uke spiste du minst 1 porsjon kjøtt eller fisk (1 porsjon er ca 150g kjøtt/fisk)?

Sett kryss for antallet dager per uke								
Matsort	***0***	***1***	***2***	***3***	***4***	***5***	***6***	***7***
Kjøtt								
Fisk								

Har du badet følgende steder i løpet av tidsperioden?

Sted	***Ja***	***Nei***
Ferskvann		
Saltvann		

Omtrent hvor mange urinveisinfeksjoner hadde du i løpet av tidsperioden?

Antall:	

UTENLANDSREISER (varighet >24 timer)

Fyll inn hvor du reiste, hvilken måned du kom tilbake og antallet dager reisen varte i den aktuelle tidsperioden

Reise nr	***Reisen gikk til (land)***	***I hvilken måned i tidsperioden kom du tilbake fra reisen?***	***Hvor mange dager varte reisen?***
1			
2			
3.			*(Benytt eventuelt et ekstra ark!)*

Kontakt med helsevesenet siste 5 år før xx

Sted	***Navn***
Fastleger jeg har brukt:	
Sykehus jeg har vært innlagt på: (>24 timer)	
Når var sist gang du var innlagt (>24 timer) på et sykehus/sykehjem/annen helseinstitusjon (også utenfor tidsperioden)	

OPPLYSNINGER OM EVENTUELL INFEKSJON DU HADDE PÅ PRØVETIDSPUNKTET (altså xx)

Hvis du ikke kan huske at du hadde en urinveisinfeksjon på det aktuelle tidspunktet kan du hoppe over dette punktet

Spørsmål (kryss av)	Ja	Nei	Spørsmål (kryss av)	Ja	Nei
Hadde du hyppig vannlating?			Følte du at du måtte plutselig på do?		
Hadde du smerter ved vannlating?			Hadde du smerter nederst i magen?		
Måtte du bytte antibiotikakur?			Hadde du feber?		
Hvor mange dager etter at kuren begynte tok det før du følte deg frisk?			Hvilken diagnose ga legen deg? Skriv inn diagnose:		
Hvilken dag ble du frisk (dato)					

Errata

The word “subgingival” was changed to ”supragingival” throughout the text in the thesis. The word appears in pages 37, 39, 41 and 60 in the printed version. It also appears in page 2, 4, 5, 6, 7, 9 and 10 in the manuscript of Paper II.

Papers I-III

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PLOS ONE

Risk Factors for Community-Acquired Urinary Tract Infections Caused by ESBL-Producing *Enterobacteriaceae* –A Case–Control Study in a Low Prevalence Country

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Abstract

Community-acquired urinary tract infection (CA-UTI) is the most common infection caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae,* but the clinical epidemiology of these infections in low prevalence countries is largely unknown. A population based case-control study was conducted to assess risk factors for CA-UTI caused by ESBL-producing *E. coli* or *K. pneumoniae*. The study was carried out in a source population in Eastern Norway, a country with a low prevalence of infections caused by ESBL-producing *Enterobacteriaceae*. The study population comprised 100 cases and 190 controls with CA-UTI caused by ESBL-producing and non-ESBL-producing *E. coli* or *K. pneumoniae,* respectively. The following independent risk factors of ESBL-positive UTIs were identified: Travel to Asia, The Middle East or Africa either during the past six weeks (Odds ratio (OR) = 21; 95% confidence interval (CI): 4.5–97) or during the past 6 weeks to 24 months (OR = 2.3; 95% CI: 1.1–4.4), recent use of fluoroquinolones (OR = 16; 95% CI: 3.2–80) and β-lactams (except mecillinam) (OR = 5.0; 95% CI: 2.1–12), diabetes mellitus (OR = 3.2; 95% CI: 1.0–11) and recreational freshwater swimming the past year (OR = 2.1; 95% CI: 1.0–4.0). Factors associated with decreased risk were increasing number of fish meals per week (OR = 0.68 per fish meal; 95% CI: 0.51–0.90) and age (OR = 0.89 per 5 year increase; 95% CI: 0.82–0.97). In conclusion, we have identified risk factors that elucidate mechanisms and routes for dissemination of ESBL-producing *Enterobacteriaceae* in a low prevalence country, which can be used to guide appropriate treatment of CA-UTI and targeted infection control measures.

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Introduction

During the past 15 years, we have observed a worldwide dissemination of infections caused by CTX-M extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* [1]. These infections are associated with increased mortality, morbidity, health care costs, and the need for broad-spectrum antibiotics [2]. Community-acquired urinary tract infection (CA-UTI) is the most common infection caused by ESBL-producing bacteria, but we have limited knowledge regarding the clinical epidemiology of these infections [1,3]. Most studies have focused on health care related infections and associated risk factors. Moreover, these studies have largely been based on information from medical records. Thus, information on possible risk factors not regularly noted in those records is sparse [4–20]. A large multinational survey of infections caused by ESBL-producing *Enterobacteriaceae* identified age ≥65 years, male sex and recent use of cephalosporins as independent risk factors for CA-ESBL infections [3]. However, the authors expressed a poor predictive value of their chosen model.

The present study was conducted in Norway. The yearly Norwegian nationwide antimicrobial resistance surveillance programme has shown a very low prevalence of infections caused by ESBL-producing *Enterobacteriaceae* [21]. A prevalence of 1.6% ESBL positive UTI in the Norwegian population was estimated for 2011. The prevalence is slowly increasing. A country with low prevalence of infections with ESBL-producing bacteria is well suited to identify risk factors for acquisition of ESBL, and a nationwide prescription database makes Norway suitable for the study of antibiotic use in detail [22]. Based on these advantages and patient interviews we aimed to investigate whether patients with ESBL positive CA-UTI have a different frequency of risk factors of CA-UTI as compared to patients with ESBL negative CA-UTI.

Materials and Methods

Design and Study Population

A case-control study was conducted at the Department of Medical Microbiology, Vestre Viken Hospital Trust situated in a mixed urban, suburban and rural area in the South-Eastern part of Norway. Our two laboratories analyse samples from in- and outpatients in an area comprising four hospitals and approximately 450.000 inhabitants (source population). The inclusion period was from February 2009 to April 2011.

The eligible population constituted all patients ≥18 years old with a urine culture yielding *E. coli* or *K. pneumoniae* >10,000 CFU/ml. The following exclusion criteria were used: i) patients who had lived in Norway for <1 year, ii) were unable to answer our questionnaire, iii) had previously diagnosed infection caused by ESBL-producing bacteria, and iv) patients with health care associated UTI (i.e., hospitalized or residing in a nursing home for >24 hours during the last 31 days).

The study population consisted of all patients willing to participate with ESBL-positive UTI (case group) and randomly selected patients with ESBL-negative UTI (control group) (Figure 1).

The patients received written information and were invited to participate by ordinary mail. Non-responders were contacted twice. Acceptance was given by returning a signed consent form.

Ethics statement. The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (reference number: 2009/2037 BS-08901b).

Data Collection

Urine cultivation and bacterial identification were performed using ChromID CPS3 agar and the VITEK-2 system (both BioMerieux, Marcy l'Etoile, France). Antimicrobial susceptibility testing and interpretations including ESBL screening were performed using VITEK-2 or agar disc diffusion method according to EUCAST recommendations and clinical breakpoints [23].

Isolates resistant to cefpodoxime, cefotaxime or ceftazidime were selected for confirmatory ESBL testing using the E-test system (AB-Biodisk, BioMerieux). ESBL genotype analysis was performed using PCR for $bla_{\text{CTX-M}}$ detection and group assignment, as described [24]. Isolates negative for $bla_{\text{CTX-M}}$ were analyzed using conventional bla_{TEM} and bla_{SHV} PCR and sequencing, as described [25].

A structured interview was performed by a trained investigator by telephone or in-person for community-based and hospitalized patients, respectively. The questionnaire was sent to the participants in advance and included questions regarding the infection for which they were included in the study, health condition (Charlson Comorbidity Index [26]), contact with the health care system in Norway and abroad (time and duration during the past 5 years), UTIs, antibiotic use, compliance with antibiotic prescriptions, prostate disease, use of a urinary catheter during the past year, oral and digestive health problems, international travel or residency lasting ≥24 hours during the past five years (time since returning home, duration and country), profession, personal hygiene, household members, pets, eating habits (meals per week of different foods and meals outside home), and recreational swimming during the past year (location, number of times and submergence of head).

In Norway antibiotics are available on prescription only. Date, type and amount of antibiotic dispensed during the past five years were obtained from The Norwegian Prescription Database [22]. Information about antibiotic use during hospitalization was obtained from medical records.

Information on previous infections with ESBL-producing bacteria was obtained from our laboratoryˋs computer system.

Statistical Analysis

This case-control study was analysed using a pragmatic strategy, which means that priority was not given to a specific hypothesis.

Univariate analyses were performed using Student's *t* test, Pearson's chi-square test or Fisher's exact test when appropriate. The association between potential risk factors and infection caused by ESBL-producing *E. coli* or *K. pneumoniae* was quantified by odds ratio (OR) with 95% confidence interval (CI). Any variable with a $p<0.15$ from the univariate analysis was considered a candidate for the multivariate model. A manual backward stepwise

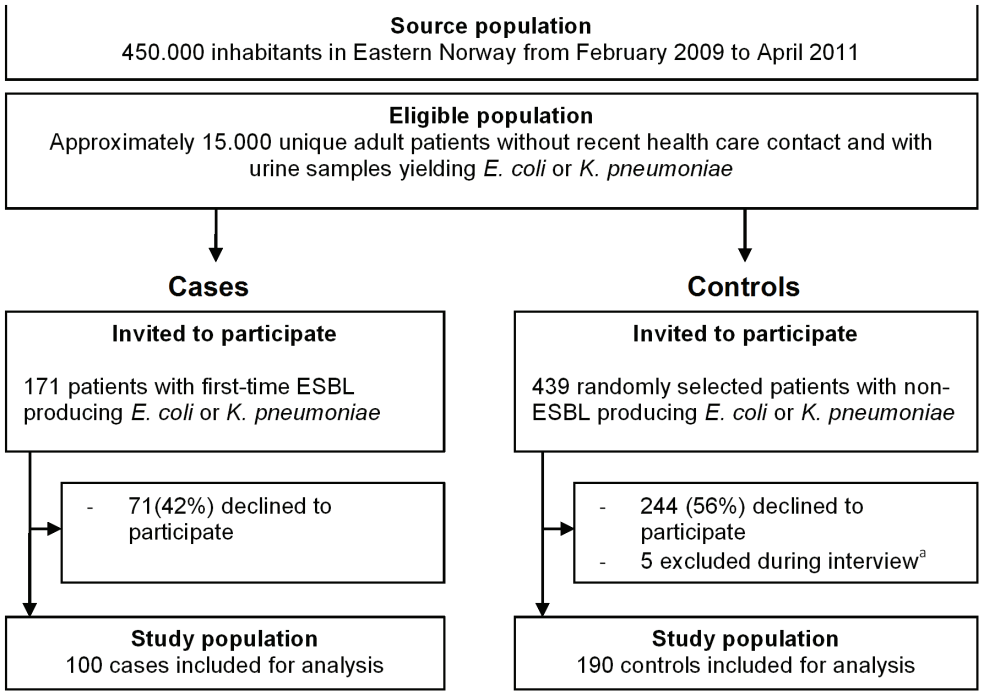

Figure 1. Selection of study population. [a]Dementia (n = 1), unable to reach by phone (n = 2) and death (n = 2).
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elimination procedure using a multivariate logistic regression model was performed to identify independent risk factors. Multivariate analyses were preceded by estimation of correlation between risk factors. Evaluation of the predictive accuracy of the models was assessed by calibration and discrimination. Calibration was evaluated by the Hosmer and Lemeshow goodness-of-fit test. A statistically non-significant Hosmer and Lemeshow result ($p>0.05$) suggests that the model predicts accurately on average. Discrimination was evaluated by analysis of the area under the ROC curve. We defined acceptable discriminatory capability as an area under the ROC curve greater than 0.7 [27]. Two-tailed *p* values of <0.05 were considered statistically significant. All statistical analyses were conducted using PASW statistics software, version 19.0 (IBM SPSS, Chicago, IL).

Results

Approximately 28,000 urine samples from 15,000 unique patients were submitted to our department during the inclusion period. A total of 359 (1.3%) samples yielded ESBL positive *E. coli* (n = 342) or *K. pneumoniae* (n = 17). After exclusion 171 subjects with ESBL UTI were invited to participate (case group). Also, 439 randomly selected control patients were invited to participate (Figure 1).

Relevant background characteristics of the participants are presented in Table 1. The cases and controls were in large similar. Significantly younger age and the presence of diabetes mellitus among cases were the two exceptions.

ESBL Genotyping

PCR and sequence analyses showed that 65%, 30%, and 5% of the ESBL isolates belonged to the CTX-M group 1, CTX-M group 9 and SHV group 5/12, respectively. TEM-type ESBLs were not detected.

Antibiotic Use and Antibiotic Resistance

Data on antibiotic use are presented in Table 2. More than 90% of the participants reported that they had completed all prescribed courses of antibiotics received during the past 5 years. Antibiotic use was more prevalent in the study population (59% during the past three months before the infection) than in the age-adjusted general Norwegian population (29% during the past year) – (data from the Norwegian Prescription Registry [22]). This difference was mainly due to increased use of antimicrobials used to treat UTIs in the study population.

In general, ESBL-producing isolates expressed more co-resistances compared to non-ESBL strains. For cases and controls the proportion of non-susceptible strains were 59% and 13% for ciprofloxacin, 78% and 24% for trimethoprim, 35% and 4% for gentamicin, 4% and 2% for nitrofurantoin and 4% and 3% for mecillinam, respectively.

Table 1. Demographic and clinical characteristics of the study population with and without ESBL positive urinary tract infection.[a]

Variable[b]	ESBL positive (n = 100)	ESBL negative (n = 190)	Crude OR	95% CI	p
Age in years, mean ± SD	55±19	64±17			<0.001
Female gender	88 (88%)	168 (88%)	0.96	0.45–2.0	0.92
Number of household members, mean ± SD	2.4±1.3	2.1±1.1			0.09
Pets in household	30 (30%)	44 (23%)	1.4	0.82–2.5	0.20
Infection caused by *Klebsiella pneumoniae*	5 (5%)	13 (7%)	0.72	0.25–2.1	0.54
Hospitalization past year[c]	21 (21%)	34 (18%)	1.2	0.66–2.2	0.52
Recurrent UTI[d]	17 (17%)	47 (25%)	0.62	0.34–1.2	0.13
Charlson index score ≥3	11 (11%)	25 (13%)	0.83	0.39–1.8	0.64
Pulmonary disease	13 (13%)	25 (13%)	0.99	0.48–2.0	0.98
Rheumatic disease	9 (9%)	33 (17%)	0.47	0.21–1.0	0.05
Malignancy	6 (6%)	9 (5%)	1.3	0.45–3.7	0.64
Diabetes mellitus	12 (12%)	9 (5%)	2.7	1.1–6.8	0.02
Gastrointestinal disease	14 (14%)	29 (15%)	0.90	0.45–1.8	0.76
Cardiac disease	13 (13%)	31 (17%)	0.76	0.38–1.5	0.44
Renal dysfunction	7 (7%)	10 (5%)	1.35	0.50–3.7	0.56
Hepatic dysfunction	1 (1%)	1 (1%)	1.90	0.12–31	1.00
Cerebrovascular disease	2 (2%)	10 (5%)	0.36	0.08–1.7	0.23
Urinary catheter at any time during past year	15 (15%)	25 (14%)	1.1	0.56–2.2	0.74

[a]Data are presented as the absolute number of patients with percentages in parentheses with the exception of age and household members, which is listed as mean value ± standard deviation (SD).
[b]Some variables have missing values (number of missing patients in parentheses): Household members (2), Charlson comorbidity index score (8) Pulmonary disease (2) Rheumatic disease (1), Malignancy (2), Diseases of the gastrointestinal tract (1), Cardiac disease (4), Renal dysfunction (1), Hepatic dysfunction (1), Cerebrovascular disease (2), Urinary catheter (6).
[c]Excluding the time period from 24 hours to 31 days before the urinary sample was taken. No patient had resided in a nursing home without being hospitalized in the time period.
[d]To quantify the number of UTIs for each patient in the preceding year, the number of prescriptions of three antimicrobial agents–trimethoprim, mecillinam, and nitrofurantoin–were counted. In Norway, these agents are first choices for UTI treatment and are not used for other infections. Recurrent UTI was defined as ≥3 UTIs during the past year.
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Table 2. Comparison of the antibiotic usage during the last 90 days prior to inclusion in the study population with and without ESBL positive urinary tract infection.

Antimicrobial agents[a]	ESBL positive (n = 100)	ESBL negative (n = 190)	Crude OR	95% CI	p
No antibiotic past 90 days[b]	38 (38%)	80 (42%)	0.84	0.51–1.4	0.50
Mecillinam	15 (15%)	45 (24%)	0.57	0.30–1.1	0.08
Macrolides	7 (7%)	5 (3%)	2.8	0.86–9.0	0.12
Tetracyclines	5 (5%)	6 (3%)	1.6	0.48–5.4	0.52
Fluoroquinolones	14 (14%)	3 (2%)	10	2.84–36	<0.001
Nitrofurantoin	8 (8%)	16 (8%)	0.95	0.39–2.3	0.90
Trimethoprim or trimethoprim/sulfamethoxazole	16 (16%)	42 (22%)	0.67	0.36–1.3	0.22
β-lactams except mecillinam[c]	18 (18%)	18 (9%)	2.1	1.0–4.2	0.04
- Phenoxymethylpenicillin	11 (11%)	12 (6%)	1.8	0.78–4.3	0.16
- Amoxicillin	3 (3%)	6 (3%)	0.95	0.23–3.9	1.0
- Cloxacillin	3 (3%)	1 (1%)	5.8	0.60–57	0.12
- Cephalexin	4 (4%)	2 (1%)	3.9	0.70–22	0.19
Methenamine hippurate	2 (2%)	15 (8%)	0.24	0.05–1.1	0.04

[a]Number of subjects who had used at least one dose in the past 90 days.
[b]Six cases and 17 controls received an antimicrobial agent at the day before the urinary sample only.
[c]Penicillin, amoxicillin, cloxacillin or cephalexin (some patients used more than one type).
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Risk Factor Analysis

The results of the univariate analyses on risk factors are presented in Table 3. Travelling to Asia, Middle East or Africa up to 2 years in the past, recreational swimming, eating dinner at restaurants and close occupational contact with humans were identified as significant risk factors for ESBL UTI. Interestingly, frequent consumption of fish meals (Figure 2), infrequent bath or shower and digestive problems seemed to have a protective effect.

The results of the multivariate analyses are presented in Table 4. Patients with an ESBL positive UTI had travelled 21 times more to Asia, Middle East or Africa during the past 6 weeks than patients with a non-ESBL UTI, and this was the strongest predictor for ESBL UTI. Travel to the same areas in the period from 6 weeks to 24 months in the past was to a lesser degree associated with ESBL UTI (OR 2.3, 95% CI: 1.2–4.4, p = 0.017). The variables regarding (time since) travel abroad were also analysed as continuous variables but this did not influence the results. Recreational freshwater swimming was identified as an independent risk factor, and patients with ESBL UTI had swum twice as frequent in freshwater as patients with ESBL negative UTI.

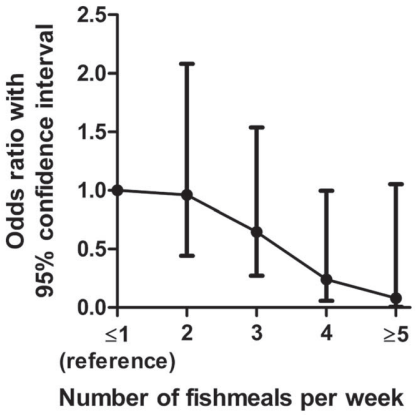

Figure 2. Decreasing risk[a] of ESBL-positive urinary tract infection with increasing number of fishmeals per week[b]. [a]Controlling for the variables: Travelling to Asia, Middle east or Africa, Use of fluoroquinolones the past 90 days, Use of β-lactams except mecillinam the past 90 days, Diabetes mellitus,Recreational freshwater swim past year and age. [b]Reference category: eating ≤1 fishmeal per week.
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Previously known risk factors such as recent antibiotic use and diabetes mellitus were also identified as independent risk factors. Age and weekly fish meals were found to be putative protective factors.

The final multivariate model was applied to participants with infection caused by *E. coli* only and this did not change any trends in the results (data not shown).

The Hosmer and Lemeshow goodness-of-fit test was not significant indicating a satisfactory fit of the model ($\chi^2 = 5.64$, df = 8, $p = 0.69$). The area under the ROC curve was 0.83 (95% CI: 0.79–0.88) indicating a good discriminative ability between ESBL-positive and ESBL-negative patients.

Discussion

This is to our knowledge the first population-based study to identify risk factors for acquisition of CA-ESBL infections in a low prevalence country. International travel was identified as the most important risk factor for ESBL positive CA-UTI in this study. Most travel-associated ESBL UTIs occurred during the first six weeks after returning home. This observation is consistent with previous studies and adds new information about the time course between colonization during travel and actual infection [5,28,29]. The area associated with the highest risk (Asia, Middle East and Africa) corresponds well with areas previously associated with a high rate of colonization in returning travellers [28].

This observation contrasts a recent French study. Nicolas-Chanoine and co-workers did not identify travelling abroad for

Table 3. Univariate comparison of risk factor exposition in the study population with and without ESBL-positive urinary tract infection.[a]

Variable[b]	ESBL positive (n = 100)	ESBL negative (n = 190)	Crude OR	95% CI	p
Travel destinations abroad within the past 6 weeks[c]					
- America or Oceania (including Japan)	0 (0%)	1 (1%)	0.65		1.00
- Asia, Middle East or Africa	23 (23%)	2 (1%)	28	6.5–122	<0.001
- Europe	11 (11%)	13 (7%)	1.7	0.72–3.9	0.22
Travel destinations abroad between the previous 6 weeks to 24 months[c]					
- America or Oceania (including Japan)	13 (13%)	17 (8.9%)	1.5	0.71–3.3	0.28
- Asia, Middle East or Africa	39 (39%)	36 (19%)	2.7	1.6–4.7	<0.001
- Europe	67 (67%)	108 (57%)	1.5	0.93–2.6	0.09
Travel destinations abroad between the previous 24 months to 5 years[c]					
- America or Oceania (including Japan)	10 (10%)	15 (7.9%)	1.3	0.56–3.0	0.54
- Asia, Middle East or Africa	26 (26%)	38 (20%)	1.4	0.79–2.5	0.24
- Europe	55 (55%)	92 (48%)	1.3	0.8–2.1	0.29
Recreational swimming past year					
- In seawater	68 (68%)	98 (52%)	2.0	1.2–3.3	0.01
- In freshwater	26 (26%)	30 (16%)	1.9	1.0–3.4	0.04
- In swimming pool	53 (53%)	78 (41%)	1.6	0.99–2.6	0.05
- Usually submerges head during recreational swimming	41 (41%)	56 (30%)	1.6	0.97–2.7	0.06
Eating habits					
- Number of fish meals per week, mean ±SD	2.1±1.1	2.7±1.4	0.67	0.54–0.83	<0.001
- Number of meat meals per week, mean ±SD	3.5±1.4	3.3±1.3	1.1	0.94–1.3	0.22
- Organic food ≥1/week	24 (24%)	40 (22%)	1.2	0.66–2.1	0.58
- Dinner at a restaurant ≥2/month	29 (29%)	28 (15%)	2.4	1.3–4.3	0.003
- Prefers meat well done	33 (34%)	74 (40%)	0.77	0.46–1.3	0.33
Close occupational contact with humans[d]	29 (29%)	31 (17%)	2.1	1.2–3.7	0.01
Bath or shower ≤2 times/week	12 (12%)	44 (23%)	0.46	0.23–0.92	0.03
Oral/dental health problems	13 (13%)	28 (15%)	0.85	0.42–1.7	0.65
Digestive problems (constipation or diarrhoea)	25 (26%)	75 (40%)	0.51	0.30–0.87	0.01

[a]Data are presented as the absolute number of patients with percentages in parentheses with the exception of fish and meat meals, which is listed as mean value ± SD.
[b]Some variables have missing values (number of missing patients in parentheses). Usually submerges head during recreational swimming (7), Organic food (7), Dinner in restaurant (2), Prefers meat well done (8), Close occupational contact with humans (6), Bath or shower (3), Digestive problems (6).
[c]Only trips lasting >24 hours outside the Nordic countries (Norway, Denmark, Finland, Sweden and Iceland) are included.
[d]Self-reported close occupational contact with humans.
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Table 4. Independent risk factors of ESBL positive community acquired urinary tract infection identified using multivariate logistic regression analysis.

Variable	Level	Adjusted OR	95% CI	P
Travelling to Asia, Middle East or Africa[a]				
- During the past 6 weeks	yes/no	21	4.5–97	<0.001
- Between the previous 6 weeks to 24 months	yes/no	2.3	1.2–4.4	0.017
Use of fluoroquinolones the past 90 days	yes/no	16	3.2–80	<0.001
Use of β-lactams except mecillinam in the past 90 days	yes/no	5.0	2.1–12	<0.001
Diabetes mellitus	yes/no	3.2	1.0–11	0.051
Recreational freshwater swim past year	yes/no	2.1	1.0–4.3	0.040
Age	5 year increase	0.89	0.82–0.97	0.014
Number of fish meals per week	1 meal increase	0.68	0.51–0.90	0.008

[a]Only trips lasting >24 hours are included.
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>14 days during the past 6 months as a risk factor for an ESBL-positive ($bla_{CTX\text{-}M\text{-}15}$) infection in hospitalized patients [6]. In our study, travelling abroad for >14 days was a strong predictor of ESBL UTI when using $bla_{CTX\text{-}M\text{-}1}$ -positive infections as an end-point (data not shown). It is likely that the importance of travel as a risk factor will differ between the French hospitalized population and the Norwegian non-hospitalized population in our study. Also, the proportion of ESBL-producing clinical isolates of *Enterobacteriaceae* in France is higher than in Norway [30]. Therefore, travel abroad from France will not have the same relative impact on the colonization and infection rate as travelling abroad from Norway. This emphasizes the importance of investigating these risk factors in a low prevalence area.

Recent antibiotic use is a known risk factor for infections caused by ESBL-positive bacteria [3,7,8,11,31]. We found that recent use of fluoroquinolones was strongly associated with an ESBL-positive UTI, supporting the results from several other studies [7,8,31].

Interestingly, the use of mecillinam as opposed to other β-lactams, was not associated with ESBL-positive CA-UTI. This may be because the oral formulation of mecillinam, pivmecillinam, is a pro-drug with minor effects on the intestinal flora [32]. Moreover, mecillinam has a selective activity against Gram-negative bacteria and is more stable against ESBL hydrolysis compared to most penicillins [33].

Recreational swimming in freshwater was identified as an independent risk factor for ESBL UTI. ESBL-producing bacteria like *E. coli* have been detected in environmental water [34–36]. Furthermore, outbreaks of *E. coli O157:H7* have been linked to swimming in contaminated freshwater [37]. Swimming may therefore be a risk factor for intestinal colonization with *E. coli* with ESBL and any subsequent UTI may be caused by a such newly acquired ESBL-producing strain from the water [38]. This finding highlights a possible link between environmental pollution and antimicrobial resistance, but will have to be substantiated before any conclusions can be drawn [39].

Interestingly, eating fish was associated with a reduced risk of ESBL UTI (Figure 2). Each weekly fish meal reduced the risk of an ESBL positive infection with about 30%. It is clear that eating habits influence the microbial flora in the gut [40]. However, whether eating fish may affect the resistance pattern of the gut microbial flora and potentially lower the risk of ESBL UTI remains speculative and eating fish may be a marker of a more fundamental risk factor not measured.

Retail chicken meat has recently been implicated as a possible source of ESBL-colonization [41]. We did not specifically investigate this possible risk factor, but ESBL-producing bacteria have only very rarely been found in the Norwegian food chain [42].

In our study, patients infected with an ESBL-producing *E. coli* or *K. pneumoniae* were significantly younger than the control patients. In two studies with similar design but including hospitalized patients, no association between age and ESBL positive infection was found [8,43]. This suggests that the epidemiology of ESBL infections differs in Norway or among non-hospitalized patients.

Limitations

Limitations include the possibility of selection bias due to non-participation and a potential problem with differential misclassification of exposure because the interviewers were not masked to the status of the patient being a case or a control. To minimize the latter the questionnaires were sent to the participants in advance and interview training was given.

We did not use the Friedman criteria for health care acquired infections and thus patients with health care system contact during the past 2–3 months and patients catheterized the past month were included for analysis [44]. Excluding these patients (n = 30) did, however, not change any trends in the results (data not shown).

Finally, our study may overestimate the use of antibiotics as a risk factor since patients in the control group, with susceptible bacteria, may be less likely to have used antibiotics. This is because non-ESBL *E. coli* and *K. pneumoniae* are more susceptible to antibiotics than ESBL-producers, and recently treated patients with such susceptible strains are therefore less likely to show up in the control group [45].

In summary, we have addressed the knowledge gap concerning risk factors for CA-UTIs caused by ESBL-producing bacteria [3]. Previously suspected risk factors for ESBL UTI have been supported and possible new ones uncovered. Our study shows that the predictive antimicrobial resistance pattern in uropathogenic *E. coli* is heavily influenced by the country the patient has recently visited [28,46]. Thus, information on recent travel is important when treating patients with serious infections that may involve this organism. Physicians in low-prevalence countries should consider ESBL when treating UTI in patients who have visited countries in Africa, The Middle East or Asia during the past six weeks [28,46].

An association between recreational swimming and ESBL UTI was detected. Further investigation to examine the possible negative impact of environmental pollution with ESBL-producing *Enterobacteriaceae* seems warranted.

Finally, eating fish regularly was associated with a protective effect against ESBL UTI. If this is confirmed in other studies, an interesting link between diet and infection has been established.

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Author Contributions

Conceived and designed the experiments: A. Søraas A. Sundsfjord PAJ. Performed the experiments: A. Søraas. Analyzed the data: A. Søraas A. Sundsfjord IS CB PAJ. Wrote the paper: A. Søraas A. Sundsfjord IS CB PAJ.

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Extended spectrum beta-lactamase-producing bacteria are not detected in oral samples from human fecal carriers of ESBL-producing *Enterobacteriaceae*

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Abstract

Background: The prevalence of infections caused by CTX-M type extended spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-E) has rapidly increased during the past 15 years. *Enterobacteriaceae* are commonly found in the gastrointestinal tract and long-term intestinal carriage is considered important for the spread of ESBL and as a source of clinical infections. Oral biofilm such as supragingival plaque is known to contain numerous antibiotic resistance determinants and may also represent a poorly investigated site for ESBL carriage and further spread.

Objective: To investigate possible carriage of ESBL-producing bacteria in supragingival plaque.

Design: We screened for the presence of aerobic and anaerobic ESBL-producing bacteria and $bla_{\text{CTX-M}}$ in supragingival plaque samples from healthy human adults with fecal carriage of CTX-M-producing *Escherichia coli*. The presence or absence of *Enterobacteriaceae* and ESBL-producing bacteria in plaque samples was evaluated using culture-based methods and consensus CTX-M PCR.

Results: Oral samples were obtained from 17 participants with known previous carriage of ESBL-producing *E. coli*. No ESBL-producing bacteria or ESBL genes were detected using culture-based and molecular methods. One *Rahnella aquatilis* harboring the class A ESBL gene $bla_{\text{RAHN-1/2}}$ was identified.

Conclusion: This pilot study supports the notion that the presence of CTX-M-producing bacteria is uncommon in oral plaque of healthy human adult fecal carriers. Due to the limited number of persons tested a low prevalence of oral ESBL-carriage in healthy adults or carriage in selected groups of patients cannot be excluded. To our knowledge, this is the first description of an *R. aquatilis* with the RAHN-1/2 gene in the oral cavity.

Introduction

The spread of genes coding for CTX-M type extended spectrum beta-lactamases (ESBLs) among *Enterobacteriaceae* has increased dramatically worldwide and is of major public health concern (1). Patients with infections caused by ESBL-producing *Enterobacteriaceae* (ESBL-E) are known to suffer from increased morbidity and mortality compared to patients with infections caused by non-ESBL-E (2). *Escherichia coli* is the most common pathogenic bacterium among *Enterobacteriaceae* in human disease and causes serious and common infections like septicemia and urinary tract infection (UTI) (3). *E. coli* is also the most commonly occurring ESBL-producing pathogen and has the gastrointestinal tract as its primary habitat (4). Secondary habitats like water and sediments are also known (4). The oral cavity is a well-known habitat of *E. coli* and other *Enterobacteriaceae,* which seem to form transient populations in this area (5,6).

The large increase in the worldwide prevalence of CTX-M type ESBL-E has been driven by horizontally transmitted genes carried by promiscuous plasmids and successful clones, but much is still unclear about the transmission of *Enterobacteriaceae* between hosts (7,8). The presence of ESBL-genes in the oral cavity, either harbored in *Enterobacteriaceae* or in other oral bacteria, might act as reservoirs of ESBL which may be shed to the intestinal tract and externally as airborne particles or through direct contact. Thus, oral carriage might influence important epidemiological parameters of ESBLs like duration of carriage and mode of transfer. While the fecal-oral route of transmission of these pathogens has been thoroughly explored in the literature less attention has been given to airborne or oral-oral transmission. *Enterobacteriaceae* survive in air and on surfaces, and carriage has been described in the oral cavity and the upper airways, thus it is possible that the impact of airborne or oral-oral transmission may have been underestimated (5,6,9-11).

Large studies have shown that the oral cavity includes several microbiological distinct niches with their own characteristic microbiota including a wide variety of resistance determinants also encoding beta-lactamases (12-17). The oral microbiota is in constant interaction with the external environment and bacteria descending to the gut must pass through this area. Thus, a two-way exchange of resistance determinants between food and drink and the oral microbiota is likely to occur (15,16). The oral cavity therefore possesses many characteristics necessary for a flexible transmission of genes, including ESBL-encoding genes from the environment to a host, between bacteria in a host or between hosts.

We aimed at determining the extent of oral carriage of ESBL in intestinal carriers of ESBL-E in a pilot study. We considered supragingival plaque the most likely oral location of ESBL-E and therefore sampled this area from known fecal carriers of *E. coli* carrying the ESBL-gene $bla_{\text{CTX-M}}$ and examined these for ESBL-producing bacteria and $bla_{\text{CTX-M}}$. To our knowledge, the prevalence of oral carriage of ESBL in known intestinal carriers of ESBL-producing bacteria has not been investigated previously.

Materials and Methods

Setting and design

As part of a study on fecal carriage of ESBL-producing *E. coli* or *Klebsiella pneumoniae* associated with UTI, supragingival plaque and repeat fecal samples were collected from known fecal carriers of these bacteria. The study was conducted at the Department of Medical Microbiology at Vestre Viken Hospital Trust in Eastern Norway and the Department of Oral Biology, University of Oslo.

The study was approved by the South-Eastern Norway Regional Committee for Medical and Health Research Ethics following the Declaration of Helsinki principles (1975, 1983) (reference number: 2009/2037 BS-08901b). Written informed consent was obtained from all participants.

Participants

The participants were adult volunteers from a study on fecal carriage of ESBL and risk factors for UTI caused by ESBL-producing *E. coli* or *K. pneumonia*e. The procedure for inclusion of participants has been described in detail previously (18,19). In brief, participants ≥ 18 years old with a fecal sample yielding ESBL-producing *E. coli* or *K. pneumoniae* after an UTI caused by one of these bacteria were considered for inclusion in the study. Eligible participants were contacted by mail and invited to donate a supragingival plaque sample.

Sample collection and analysis

Fecal samples

Fecal samples were collected by the participants themselves using a sterile cotton swab applied to the toilet paper after defecation. The sample containers were returned by ordinary mail and cultured aerobically on ESBL selective agar plates (ChromID ESBL, BioMerieux,

Marcy l'Etoile, France) and on a non-selective lactose agar plate as growth control. Species identification was based on characteristic pink to burgundy oxidase-negative colonies for *E. coli.* Species identification of green colonies was obtained using the VITEK-2 system (GN ID card, BioMerieux). Genotypic verification of ESBL and phylogroup assignment of fecal ESBL-positive isolates were performed using polymerase chain reaction (PCR) as described earlier (20). Sub-typing of fecal ESBL-producing *E. coli* isolates was performed by ten-loci multiple-locus variable-number tandem-repeat analysis (MLVA) as described earlier (21,22).

Oral plaque samples

Supragingival plaque samples were collected with sterile Gracey-designed steel curettes (Stalan GMBH, Ahrensburg, Germany) from the mesio-buccal aspect of every tooth, with one vertical stroke in participants with their own teeth intact and occurrence of periodontal disease was registered in all patients. From participants with dental prostheses or implants biofilms were collected from these restorations. The samples were immediately transferred to ≈5.5 ml of a prereduced anaerobically sterilized transport medium (Dental Transport Medium, Anaerobic System, Morgan Hill, CA), a medium in which also facultative organisms will survive. The samples were then within four hours homogenized by vortex and plated onto McConkey agar plates, ChromID ESBL (BioMerieux) and blood agar plates (nonselective trypticase soy agar supplemented with 5% defibrinated human blood, hemin 5 mg/ml, and menadione 0.05 mg/ml) and incubated at 37^{o}C for 24-48 hours in aerobic (McConkey and ChromID ESBL) and anaerobic (90% N_2, 5% H_2, 5% CO_2) conditions (blood agar plates). Species identification of suspected *Enterobacteriaceae* was obtained using the VITEK-2 system (GN ID card, BioMerieux).

For PCR 100 µl of the inoculated transport medium was diluted in 100 µl of Tris EDTA buffer and frozen at -70^{o}C. DNA was extracted using MasterPure DNA Purification Kit

(Epicentre, Madison, WI) according to the instructions of the manufacturer. PCR was performed in duplicate using the same procedure as for the fecal samples (20).

Sensitivity of PCR

To semi-quantitatively assess the sensitivity of the DNA extraction and PCR procedure described for oral plaque samples, 10-fold dilutions of CTX-M 1 producing *E. coli* (CCUG 55971) were inoculated in dental transport medium. DNA extraction and PCR were performed as described for oral samples above. The detection limit of the DNA extraction- and PCR procedures from the dental samples were found to be 3-30 gene copies per PCR run indicating a lower detection limit of approximately 1,600-16,000 ESBL gene copies per supragingival plaque sampling procedure. The approximation assumed the same copy number of $bla_{\text{CTX-M}}$ genes in the clinical isolates as in the reference strain *E. coli* (CCUG 55971).

Results

Patient characteristics and results from culture- and molecular analyses of fecal and oral samples are presented in Table 1. A total of 17 participants were enrolled in the study. The mean age was 61 years (range 23-84) and 13 (76%) participants were female.

Fecal samples

Fecal carriage of ESBL-E was a prerequisite for inclusion and therefore present in all participants. At least one follow-up fecal sample was obtained after dental sampling from 14 of 17 (82%) participants. The ESBL determinants in inclusion- and follow up samples were harbored exclusively in *E. coli* and of the 17 inclusion isolates twelve belonged to CTX-M phylogroup 1 and five to CTX-M phylogroup 9. The median duration from the inclusion sample to dental sampling was 64 days (range 8-326 days). The first follow-up sample from each participant was obtained at median 55 days (range 3-768 days) after dental sampling.

Results from 17 participants:

- In six (35%) participants the same ESBL phylogroup and MLVA profile were identified in both the inclusion and follow-up sample.
- In two (12%) participants with the same phylogroup the MLVA profile diverged in four and seven loci.
- In one (6%) participant the same phylogroup was only detected in the third fecal sample after dental sampling and at that point the MLVA profile diverged in four loci.
- Six (35%) participants did not donate a follow-up fecal sample or donated a sample more than 700 days after the dental procedure and all of these were negative.
- Two (12%) participants donated fecal samples promptly after the dental procedure, but these and later samples were CTX-M-negative.

Of the nine fecal ESBL culture-positive isolates obtained after the dental procedure seven isolates belonged to CTX-M phylogroup 1, one to CTX-M phylogroup 9 and in one participant both CTX-M 1 and 9 were identified.

Oral plaque samples

Of the 17 participants, 15 had their own teeth, one had a combination of own teeth and implants and one had a dental prosthesis only. Two patients were noted to have marginal periodontitis. All oral samples were culture-positive on the blood agar plates. No ESBL-producing oral bacteria were detected by culturing on the ChromID ESBL. *Enterobacteriaceae* were isolated from the McConkey cultures in three patients (17.6%) (from three samples from these three participants four different *Enterobacteriaceae* strains were identified) whereof none were noted to have periodontitis. The strains were identified as *Rahnella aquatilis* (n=1), *Pantoea* sp. (n=2) and *Enterobacter cloacae* (n=1).

Classical ESBLs were not identified by PCR in any of the oral plaque samples, but the *R. aquatilis* strain was revealed to harbor the class A ESBL gene $bla_{\text{RAHN-1/2}}$ by PCR (23).

Discussion

We have investigated the oral cavity as a potential reservoir for the CTX-M type ESBL resistance determinants in adults with fecal carriage of CTX-M producing *E. coli* using culture-based and molecular methods. The negative results obtained do not support the oral cavity as a significant reservoir of CTX-M type ESBL.

The sensitivity of the molecular methods and ChromID ESBL agar plates used for detection of ESBL-E is high (24). Furthermore, we sampled supragingival plaque because this is the oral location we considered most likely to harbor *Enterobacteriaceae*. Thus, ESBL-producing strains or $bla_{\text{CTX-M}}$ should have been detected, if present in the oral samples from our participants. The negative results indicate absence or presence in low concentrations only, of oral ESBLs in intestinal carriers. The negative PCR results also indicate that classical CTX-M genes are not harbored in any of the many different oral bacterial species present in oral samples.

Our result on oral carriage elucidates the difference between fecal and oral carriage of ESBL-E. A recent study by Titelman et al. investigated the duration of fecal carriage after ESBL-E infections and found a gastrointestinal carriage rate of 66% three months after, and 43% one year after infection (25). The difference between oral and fecal carriage duration is probably a result of the oral cavity being a microbiological site distinctly different from the gastrointestinal tract. In one study 509 different bacteria were detected in periodontal samples, but only 29 of these were found among the 560 intestinal- or fecal bacteria isolated, illustrating this point (26). Furthermore, to our knowledge only narrow spectrum beta-lactamases, AmpC beta-lactamases or unspecified TEM-genes have been detected in supragingival plaque of patients with periodontitis or in dental samples from the normal population (17,27-29). The *R. aquatilis* with the ESBL gene $bla_{\text{RAHN-1/2}}$ identified in the present material is thus, to our knowledge, a novelty. However, *R. aquatilis* is probably a transient bacterium in the mouth originating from drinking water (30). Supporting this, it was

not identified in the patient's fecal samples, and this finding does not change the overall picture of intestinal ESBL carriage as not related to oral carriage.

The present study does not support the oral cavity as a source of transmission of ESBL. But worryingly, after doxycycline therapy more than a 10-fold increase was seen in supragingival numbers of *Enterobacter aerogenes* and *E. coli* (31). Another study showed an increasing frequency of nasal and pharyngeal carriage of *Enterobacteriaceae* during the hospitalized period in patients admitted to general wards and intensive care units (10). Thus, ESBL-E may under certain circumstances be more frequent in the oropharyngeal area than our study demonstrated. Possible transmission related to oropharyngeal carriage was demonstrated in another study by Branger et al. which identified an outbreak of ESBL-E possibly related to a poorly maintained bronchoscope (32). Thus, there are suggestions that periods of oral- or upper airway carriage may facilitate transfer of ESBL-E, but this remains to be documented. In hospitals, where oropharyngeal carriage appears to be more frequent, this may have serious consequences due to inter-patient transmission and the present results should not discourage infectious control measures that take into account a possible oropharyngeal source of ESBL-E.

Our study has limitations of which the most important is its small size. Furthermore, the intestinal colonization status of the participants at the exact time of the dental sampling procedure was unknown since fecal samples were not collected at that time. However, ESBL was detected in 9 of 11 (82%) participants that donated a fecal sample within 90 days after dental sampling. This is in line with published data on the duration of fecal ESBL carriage after infection (25). The exact same ESBL- or *E. coli* sub-type was not identified in all nine participants. This may be explained by re-colonization and thus it is a possibility that these participants were not fecal carriers at the time of the dental procedure. Since Norway is a country with a low prevalence of ESBL, this seems unlikely. Other explanations like initial co-colonization with several ESBLs, transfer of plasmids between *E. coli* strains and ESBL

below the detection threshold seem better explanations for diverging phylogroups and sub-types before and after dental sampling.

It's not possible to know how many participants were actually fecal carriers at the time of oral sampling, but a crude and somewhat optimistic estimate may be 82% of 17 (=14). This would give us above 95% chance of detecting a 25% oral carriage rate.

However, none of 17 previously known fecal carriers of ESBL were found to be oral carriers at a median of 64 days after fecal sampling. At this point in time at least 66% of patients infected with ESBL-E would be expected to be fecal carriers (25). Thus, we may make a cautious conclusion that the course of fecal carriage is significantly different and more prolonged than oral carriage in our participants ($p<0.001$). The results do not rule out transient oral ESBL-carriage in fecal carriers. Because of the limitations of this pilot study and the importance of the issue investigated further studies of oral carriage of ESBL are warranted.

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Table 1.

Background information about participants and time of fecal sample before and after the oral sampling procedure and results from these samples.

ID	Age	Gender (F/M)	Number of days from collection of fecal sample to collection of oral sample	Number of days from oral sampling to next fecal sample	CTX-M genotype of *E. coli* from fecal sample before oral sampling [a]	CTX-M genotype of *E. coli* from first fecal sample obtained after oral sampling [a]	MLVA results[c] (number of diverging loci before and after dental sampling)
1	64	M	8	81	M1	M1	0
2	65	F	11	81	M1	M1	0
3	73	F	11	80	M1	M1	7
4	45	F	14	90	M1	M1	0
5	74	F	38	-	M1	-	NA
6	52	F	44	13	M9	M9	0
7	23	F	56	16	M1	M1	4
8	84	F	57	13	M1	Negative	NA
9	69	F	64	6	M1	M1	0
10	50	F	71	17	M1	Negative	NA
11	68	M	74	726	M9	Negative	NA
12	70	M	77	706	M9	Negative	NA
13	59	M	100	-	M1	-	NA
14	42	F	122	768	M1	Negative	NA
15	75	F	148	29	M1	M1	0
16	45	F	186	3	M9	Negative [b]	4
17	79	F	326	-	M9	-	NA

[a] All the CTX-M genes identified prior to, and directly after oral sampling were harbored in *E. coli*

[b] 97 days later CTX-M 1 was detected and 145 days later CTX-M 1 and 9 were detected

[c] "NA" indicates that ten-loci multiple-locus variable-number tandem-repeat analysis (MLVA) was not performed.

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High Rate of Per Oral Mecillinam Treatment Failure in Community-Acquired Urinary Tract Infections Caused by ESBL-Producing *Escherichia coli*

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Abstract

A population-based study was performed to investigate the efficacy of mecillinam treatment of community-acquired urinary tract infections (CA-UTI) caused by extended-spectrum β-lactamase (ESBL) producing *Escherichia coli.* The study was conducted in South-Eastern Norway. Data from patients with CA-UTI caused by ESBL-producing and non-producing (random controls) *E. coli* were collected through interviews, questionnaires, medical records and the Norwegian Prescription Database. Treatment failure was defined as a new antibiotic prescription appropriate for UTI prescribed within two weeks after the initial antimicrobial therapy. Multivariable logistic regression analysis was performed to identify treatment agents and patient- or bacterial traits associated with treatment failure. A total of 343 patients (mean age 59) were included, of which 158 (46%) were treated with mecillinam. Eighty-one patients (24%, mean age 54) had infections caused by ESBL producing *E. coli,* and 41 of these patients (51%) received mecillinam as the primary treatment. Mecillinam treatment failure was observed in 18 (44%) of patients infected by ESBL-producing strains and in 16 (14%) of patients with a CA-UTI caused by ESBL non-producing strains. Multivariable analysis showed that ESBL status (odds ratio (OR) 3.2, 95% confidence interval (CI) 1.3–7.8, $p = 0.009$) and increased MIC of mecillinam (OR 2.0 for each doubling value of MIC, CI 1.4–3.0, $p<0.001$) were independently associated with mecillinam treatment failure. This study showed a high rate of mecillinam treatment failure in CA-UTIs caused by ESBL producing *E. coli*. The high failure rate could not be explained by the increased MIC of mecillinam alone. Further studies addressing the use of mecillinam against ESBL-producing *E. coli,* with emphasis on optimal dosing and combination therapy with β-lactamase inhibitors, are warranted.

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Introduction

Escherichia coli is the most common cause of community-acquired urinary tract infection (CA-UTI). The worldwide dissemination of multidrug resistant CTX-M extended spectrum β-lactamase (ESBL)-producing *E. coli* has significantly limited the oral treatment options for CA-UTI [1]. Mecillinam is an amidinopenicillin with selective activity against Gram-negative bacteria and *Enterobacteriaceae* in particular. It is widely used in the Scandinavian countries, but the guidelines regarding dosage varies between countries with 200 mg thrice daily (TID) usually prescribed in Norway. In vitro data suggest that mecillinam has a favourable stability to β-lactamase hydrolysis compared with other penicillins [2]. International treatment guidelines endorse the use of mecillinam with an A1-grading of recommendation as a first choice treatment for uncomplicated lower urinary tract infection in women [3]. Mecillinam can be administered per os as a prodrug, the pivaloyloxymethyl ester pivmecillinam, which after absorption is converted to the antibacterial active mecillinam [4]. Mecillinam has been shown to exert a minor ecological impact on the human commensal flora [5,6]. The favourable ecological profile is also underlined by the observed stable and low (<2%) rate of resistance to mecillinam in uropathogenic *E. coli* in repeated international surveys as well as in Scandinavian countries with a widespread use of pivmecillinam over many years [7,8].

In vitro antimicrobial susceptibility tests have provided favourable results for mecillinam against CTX-M producing *E. coli* [9–12]. However, a clavulanate reversible inoculum dependent effect that significantly increases the minimum inhibitory concentration (MIC) for mecillinam in ESBL-producing *E. coli* compared to non-producers has been reported [13,14]. Titelman et. al also found a low bacteriological cure rate (two of eight patients) in a recent study and these notions underline the need for studies addressing the clinical efficacy of mecillinam in CA-UTI caused by ESBL producing *Enterobacteriaceae* [10]. To our knowledge only case-report studies have been reported so far [10,15].

In this population-based study we aimed to prospectively examine the clinical efficacy of mecillinam in the treatment of CA-UTI caused by ESBL-producing *E. coli* compared to non-ESBL-producing *E. coli.*

Patients and Methods

Ethics Statement

The study was approved by the Regional Committee for Medical and Health Research Ethics – South East ("REC South East"), following the Declaration of Helsinki principles (reference number: 2009/2037 BS-08901b). It is registered in ClinicalTrials.gov (Identifier: NCT01838213).

Setting and Design

This study was part of an investigation of CA-UTI and faecal carriage of ESBL-producing bacteria conducted in Norway at the Department of Medical Microbiology, Vestre Viken Hospital Trust, between February 2009 and May 2012 [16]. The hospital trust serves approximately 450.000 inhabitants and is situated in a mixed urban, suburban and rural area in the South-Eastern part of the country. Our two laboratories analyze in- and outpatient samples from this area.

Patients with any type of CA-UTI caused by ESBL-producing or non-ESBL producing *E. coli* and receiving empirical treatment were included in the study. Data on treatment outcome were obtained and possible associations between outcome and mecillinam treatment, ESBL-status and other variables were investigated.

Participants

The eligible population constituted all patients ≥18 years old with a urine culture yielding *E. coli* >10,000 CFU/ml. We excluded patients who: i) had not been empirically treated (i.e., did not collect an antimicrobial agent appropriate for UTI (trimethoprim, trimethoprim-sulfamethoxazole, ciprofloxacin, ofloxacin, nitrofurantoin, pivmecillinam, amoxicillin or cephalexin) at a Norwegian pharmacy at the index date (fosfomycin and amoxicillin/clavulanate are not available in Norway), ii) had lived in Norway for <1 year, iii) were unable to answer the questionnaire, iv) had previously diagnosed infection caused by ESBL-producing bacteria, or v) had health care associated UTI (i.e., had been hospitalized or residing in a nursing home for >24 hours during the last 31 days).

Procedures for inclusion of participants and data collection have been described earlier [16]. In brief, participation required a written consent; all eligible patients with an ESBL-producing *E. coli* were invited to participate. For each patient with an ESBL-producing *E. coli* invited, 2–5 patients with non-ESBL *E. coli* urine isolates during the same time period were randomly selected (Excel® randomization, Microsoft, Redmond, WA) and invited to participate. Participants answered standardized questionnaires which included queries about the current UTI, previous UTIs, contact with the health care system, catheter use and adherence to antibiotic prescriptions. Detailed data about antimicrobial drugs dispensed were collected from The Norwegian Prescription Database and from medical records [17]. To quantify the number of UTIs for each patient in the preceding year, the number of prescriptions of three antimicrobial agents–trimethoprim, mecillinam, and nitrofurantoin–were counted in individual patients. In Norway, these agents are first choices for UTI treatment and are not prescribed for other infections.

Microbiological Data and Antibiotic Susceptibility

Urine cultivation and bacterial identification were performed using ChromID CPS3 agar and the VITEK-2 system (both BioMerieux, Marcy l'Etoile, France). Antimicrobial susceptibility testing and interpretations including ESBL screening were performed using VITEK-2 (AST- N029, N122 or N209 card) which reports MIC of mecillinam in categories ≤1, 2, 4, 8, 16, 32 and ≥64 mg/L based on measurements in wells with mecillinam concentrations of 1, 3, 8 and 32 mg/L. All isolates resistant to cefpodoxime, cefotaxime or ceftazidime were selected for confirmatory ESBL testing using the Etest gradient system (AB-Biodisk, BioMerieux). Clinical breakpoint interpretations were according to EUCAST. The breakpoint for resistance for mecillinam in *E. coli* was >8 mg/L during the study period [18].

Molecular Detection of ESBL

ESBL genotype analysis was performed using PCR for $bla_{\text{CTX-M}}$ detection and group assignment, as described [19]. Detection of bla_{TEM} and bla_{SHV} was performed on ESBL-positive isolates negative for $bla_{\text{CTX-M}}$ using consensus PCR followed by DNA sequencing [20].

Treatment Outcome Measures

Two different treatment failure measures were obtained and compared: i) a patient receiving a second antibiotic prescription appropriate for UTI (same antibiotics as in inclusion) within day 1–14 after the index date [21–23] ii) a patient reporting not to have been subjectively cured within 14 days after initial treatment.

Statistical Analysis

The statistical analyses were conducted using PASW statistics software, version 19.0 (IBM SPSS, Chicago, IL). Univariate analyses were performed using logistic regression, Pearson chi square, Fisher's exact test, Student's t-test or the Mann-Whitney U-test as appropriate. The association between variables and treatment failure was quantified by odds ratio (OR) with 95% confidence interval (CI). Variables with a $p<0.15$ were considered candidates for the multivariable model. A manual backward stepwise elimination procedure using multivariable logistic regression was performed to identify independent risk factors for treatment failure. Multivariable analyses were preceded by estimation of correlation between risk factors and followed by testing of all initial variables added to the final model. All p-values were two-tailed, and a p-value of <0.05 was considered significant. The two outcome measures were compared using Cohens kappa.

Results

A total of 478 (1.5%) of approximately 32.000 urine samples analysed during the inclusion period yielded an ESBL-producing *E. coli*. Of these 478 samples, 231 (48%) were from ineligible patients (mostly because of earlier ESBL and contact with the health care system) and 247 (52%) were from eligible patients. Of these, 132 (53%) consented to participate, but 49 (37%) had not received an antimicrobial at index date and 2 were ineligible for other reasons leaving 81 participants. Among 1330 randomly selected patients with non-ESBL UTI, 453 (34%) consented to participate. Of these, 185 (41%) had not received an antimicrobial drug at index date and six were ineligible for other reasons leaving 262 participants with non-ESBL UTI.

Participants

The study population had a mean age of 59 years (range 18–93 years), which was comparable to that of all patients invited to participate (62 years). In total, 87% of the participants were female. The mean age of patients with an ESBL-producing *E. coli* was 54 years (range 18–92 years), which was significantly younger than patients with a non-ESBL-producing strain (61 years). There were no significant differences between patients with ESBL-positive or ESBL-negative UTI in relation to gender, prescribed

treatment (type or duration) or number of urinary tract infections during the past year.

Antibiotic Susceptibility

An overall higher prevalence of antimicrobial-resistance was detected in ESBL-producing strains than in non-producers (Table 1). The MIC of mecillinam in ESBL-producing strains was higher than in non-ESBL producing strains (2 mg/L (interquartile range (IQR) 0 to 4 mg/L) vs. ≤1 mg/L (IQR ≤1 to ≤1 mg/L), $p<0.001$).

Molecular Detection of ESBL

PCR and sequence analyses showed that 68%, 28%, and 2.5% of the ESBL isolates belonged to the CTX-M group 1, CTX-M group 9 and SHV group 5/12, respectively. One ESBL-isolate was not available for ESBL-typing. TEM-type ESBLs were not detected.

Treatment Outcome Measures

Information on repeat prescriptions (interpreted as treatment failure) were available from the Norwegian Prescription Database and medical records for all participants (n = 343). Clinical data to assess the clinical outcome were available for 251 patients (73%) only. The participants with missing information on the clinical outcome were evenly distributed between the ESBL-positive and ESBL-negative groups. There was substantial agreement between the two outcome measures with Cohen's kappa = 0.70 and congruent results in 219 (87%) of cases evaluable with both methods [24]. Due to the completeness of data, results based on the prescription registry (repeat prescriptions) will be presented henceforth.

Treatment Outcome

In total, 101 (29%) treatment failures as determined by repeat prescriptions were recorded, of which 73 (72%) occurred within the first seven days after initiation of treatment. The treatment failure rate was higher among patients with an ESBL-positive strain (53%) than an ESBL-negative strain (22%) ($p<0.001$). There were no significant differences in treatment outcome between the different ESBL genotypes.

Treatment outcomes were compared between patients treated with mecillinam (mecillinam-group) and those treated with other antimicrobials (non-mecillinam group). The two groups were similar with regard to background characteristics with the exception of gender and prescribed dose. Females were given mecillinam treatment more frequently than males (49% vs. 23%, respectively, $p = 0.001$). The mean dose of antimicrobial agent dispensed for the actual UTI was 8.3 defined daily doses (DDD) in the mecillinam group as compared to 6.1 DDD in the non-mecillinam group ($p<0.001$). Approximately 75% of the patients received a prescription for seven days or more as judged from the number of DDDs. Self-reported compliance with prescribed antibiotics exceeded 90% in both treatment groups.

In the mecillinam treatment group the rate of treatment failure among patients with ESBL-producing strains was 44% vs. 14% for patients with non-ESBL producers (Figure 1). Age, the strain's ESBL status, MIC of mecillinam and overall resistance profile were associated with treatment failure (Table 2). Treatment failed in all four patients with strains that were *in vitro* resistant to mecillinam (3 ESBL-positive strains and 1 ESBL-negative strain). In contrast, we observed a much lower rate of treatment failure (20%) in patients (n = 15) with ESBL-producing strains with a low mecillinam MIC (≤1 mg/L).

In the non-mecillinam treatment group the overall prevalence of treatment failure among patients with and without ESBL-producing strains was 63% and 29%, respectively. Furthermore, the prevalence of treatment failure was 85% and 16% in patients who received an antimicrobial for which their strain was *in vitro* resistant or non-resistant, respectively. In vitro resistance to the dispensed antimicrobial agent (i.e., inappropriate initial treatment), ESBL status and overall resistance profile were associated with treatment failure (Table 3).

Multivarible Analysis

Results from the multivariable analysis are presented in Table 4. The multivariable analyses were performed separately on each of the two treatment groups.

Mecillinam treatment group. The ESBL status and the strain's MIC of mecillinam were both retained in the final model, and thus associated with treatment failure. An ESBL-producing strain was associated with a three-fold risk, and each doubling of mecillinam MIC (from ≤1 mg/L), was associated with a two-fold risk of treatment failure. Thus, the treatment failure rate for ESBL-positive strains was substantially greater than for ESBL-negative strains expressing the same mecillinam MIC (Figure 1).

Non-mecillinam treatment group. Inappropriate initial treatment was the only variable retained in the final model and was strongly associated with treatment failure. If this variable was omitted from analysis, the final model would include ESBL status (OR = 2.4, CI 1.03–5.5, p = 0.04), trimethoprim resistance

Table 1. Prevalence of resistance in ESBL-producing and non-ESBL-producing *E. coli*.

Resistance to	ESBL-producing *E. coli* (n = 81)	Non-ESBL-producing *E. coli* (n = 262)	p-value
Ampicillin	100%	40%	<0.001
Mecillinam	6.2%	0.4%	0.001
Trimethoprim	74%	29%	<0.001
Trimethoprim-sulfamethoxazole	72%	27%	<0.001
Nitrofurantoin	1.2%	0.0%	0.24
Ciprofloxacin	53%	7.7%	<0.001
Gentamicin	38%	5.2%	<0.001
Cefuroxime	98%	2.4%	<0.001
Cefotaxime	98%	0%	<0.001

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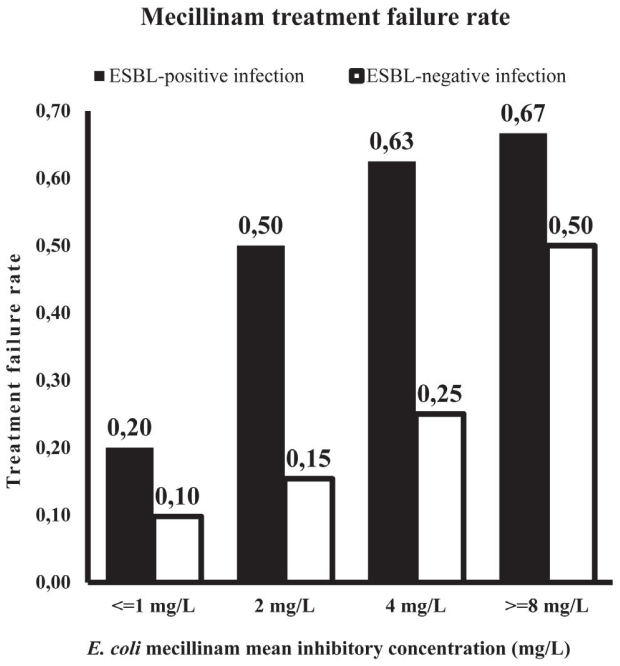

Figure 1. Mecillinam treatment failure rate among patients with community-acquired urinary tract infection caused by ESBL-producing and non-ESBL-producing *E. coli* with different mecillinam mean inhibitory concentrations.
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(OR = 6.4, CI 3.1–13.2, p<0.001) and treatment with nitrofurantoin (OR = 0.25, CI 0.08–0.8, p = 0.02).

The results of the multivariable analysis did not change significantly when i) the four patients with *in-vitro* mecillinam resistant microbes were excluded from the analysis in the mecillinam treatment group in the final model, ii) participants with a recent (≤1 month) history of UTI were excluded or iii) when the variables age, gender and number of UTIs during the past year were included in the final model.

Discussion

To our knowledge this is the first population-based study on the clinical efficacy of mecillinam in CA-UTI. We observed a significantly higher rate of mecillinam treatment failure in patients with a CA-UTI caused by ESBL-producing *E. coli* compared to non-ESBL-producing strains. The ESBL-producing strains were dominated by CTX-M type 1 and 9, in accordance with the current national and international situation [25,26].

There are several possible explanations for the high rate of mecillinam treatment failures in ESBL-producing *E. coli*. Firstly, we observed that the mean MIC of mecillinam in ESBL-producing strains was higher than in non-producers. A doubled MIC of mecillinam was associated with a two-fold risk of treatment failure in both univariate and multivariable analysis (Figure 1). This observation suggests that increasing the mecillinam dose or the dosing frequency might have reduced the treatment failure rate because the bactericidal activity of betalactam antibiotics is dependent on the time period the drug concentration exceeds the actual MIC at the infection site [27]. This notion is also supported by urine concentration measurements of mecillinam in healthy adults showing that a sensitive *E. coli* population should be suppressed by mecillinam in urine throughout a 24-h period if 400 mg pivmecillinam is given thrice daily [28]. Only 200 mg TID was prescribed to most study patients in accordance with Norwegian guidelines. Moreover, Monte Carlo simulations run to predict serum concentrations after 400 mg pivmecillinam given per os TID also support a higher dosage [29]. These simulations showed that this dose will only achieve a serum concentration above MIC for more than 40% of the time if MIC ≤0.25 mg/L. This is lower than for most ESBL-producing strains and supports the fact that that treatment failures can occur because of low dosing of mecillinam. Mecillinam and active metabolites accumulate in urine and a reduced antimicrobial potency of mecillinam would especially occur towards bacteria with slightly elevated MICs in upper urinary tract infections where

Table 2. Relevant patient characteristics in the mecillinam treatment group and univariate analysis of risk factors for treatment failure[a].

Characteristic	Treatment failure n = 34	Treatment success n = 124	Crude OR	95% CI	p-value
Age in years, mean ± SD	53±17	61±19	0.98	0.96–0.998	0.03
Female gender (%)	32 (94)	116 (94)	1.1	0.22–5.5	1.0
Number of urinary tract infections during past year, mean ± SD[b]	1.0±1.4	1.2±1.5	0.91	0.69–1.2	0.51
Total prescribed dose of antimicrobial agent in DDD (median, IQR)[c]	6.7 (6.7–10)	6.7 (6.7–10)	0.82	0.66–1.0	0.13
ESBL-producing strain (%)	18 (53)	23 (19)	4.9	2.2–11	<0.001
Mecillinam MIC (mg/L) (median IQR)[d]	2 (≤1–4)	≤1 (≤1–≤1)	1.3	1.1–1.5	<0.001
Strain resistant to initial treatment (mecillinam) (%)	4 (12)	0 (0)	–	–	0.002
Strain resistant to ampicillin (%)	26 (76)	57 (46)	3.8	1.6–9.1	0.002
Strain resistant to ciprofloxacin (%)	12 (35)	19 (16[e])	3.0	1.3–7.0	0.01

[a]Data are presented as the absolute number of patients unless specifically noted.
[b]To quantify the number of UTIs for each patient in the preceding year, the number of prescriptions of three antimicrobial agents–trimethoprim, mecillinam, and nitrofurantoin–were counted. In Norway, these agents are first choices for UTI treatment and are not used for other infections.
[c]OR is per increase of one defined daily dose (DDD) (One DDD = 600 mg of pivmecillinam), IQR = inter-quartile range.
[d]MIC = minimal inhibitory concentration.
[e]Missing information on two patients.
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Table 3. Relevant patient characteristics in the non-mecillinam treatment group and univariate analysis of risk factors for treatment failure[a].

Characteristic	Treatment failure, n = 67	Treatment success n = 118	Crude OR	95% CI	p-value
Age in years, mean ± SD	57±18	61±16	0.99	0.97–1.0	0.14
Female gender (%)	54 (81)	98 (83)	0.85	0.39–1.8	0.68
Number of urinary tract infections during past year, mean ± SD[b]	1.0±1.8	1.1±1.7	0.96	0.80–1.1	0.34
Total dispensed dose of antimicrobial agent in DDD (median, IQR)[c]	5.6 (4.0–7.0)	5.6 (4.5–6.3)	0.95	0.86–1.1	0.33
ESBL-producing strain (%)	25 (37)	15 (13)	4.1	2.0–8.5	<0.001
Strain resistant to initial treatment (%)	45 (68[d])	8 (6.8)	29	12–71	<0.001
Strain resistant to ampicillin (%)	52 (78)	50 (42)	4.7	2.4–9.3	<0.001
Strain resistant to ciprofloxacin (%)	21 (31)	11 (9.3)	4.4	2.0–10	<0.001
Strain resistant to trimethoprim (%)	46 (69)	28 (24)	7.0	3.6–13	<0.001
Treatment					
- Treated with trimethoprim (%)	41 (61)	66 (56)	1.2	0.67–2.3	0.49
- Treated with a quinolone (%)	5 (7.5)	12 (10)	0.71	0.24–2.1	0.54
- Treated with nitrofurantoin (%)	5 (7.5)	21 (18)	0.37	0.13–1.0	0.052
- Treated with another antibiotic (including combinations)[e] (%)	16 (24)	19 (16)	1.6	0.78–3.4	0.19

[a]Data are presented as the absolute number of patients unless specifically noted.
[b]To quantify the number of UTIs for each patient in the preceding year, the number of prescriptions of three antimicrobial agents–trimethoprim, mecillinam, and nitrofurantoin–were counted. In Norway, these agents are first choices for UTI treatment and are not commonly used for other infections.
[c]OR is per increase of one defined daily dose (DDD). IQR = inter-quartile range.
[d]Missing information on one patient.
[e]The other group consist of patients treated with (numbers of patients in parentheses): trimethoprim-sulfamethoxazole (16), intravenous treatment (9), amoxicillin (5), cefalexin (3), pivmecillinam and nitrofurantoin (1) and pivmecillinam and trimethoprim (1).
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accumulation of mecillinam in the urine is less pronounced [28,30,31].

The other variable found to be associated with treatment failure in multivariable analysis was ESBL-status itself (Figure 1). This is consistent with previous in vitro studies on the activity of mecillinam against ESBL-producing *E. coli* showing that mecillinam is not stable against ESBLs [2,13,14]. ESBL-producing strains have an inoculum dependent MIC for mecillinam. Agar dilution analyses of CTX-M producing *E. coli* with and without clavulanic acid added showed a significant inoculum effect on the MIC of mecillinam that was reversed by clavulanate [13,14]. An inoculum of 10^6 CFU/spot gave an approximately 100-fold increase in mecillinam MIC compared to the standard inoculum (10^4 CFU/spot). Interestingly, recently published time-kill analyses showed a significant bactericidal activity in only 7/48 (15%) of CTX-M producing *E. coli* strains even with the addition of clavulanic acid [13]. In vitro antimicrobial susceptibility tests are mostly based on bacteriostatic rather than bactericidal activity and the observed reduced bactericidal effect of mecillinam against ESBL-producing *E. coli* may therefore pass unrecognised.

Our results may seem to contradict the recently published 100% mecillinam treatment success rate in seven patients with ESBL-producing *E. coli* [10]. However, six of those strains had a MIC of mecillinam ≤1 mg/L while the last one had a MIC of 2 mg/L. Among our fifteen ESBL-producing strains with a mecillinam MIC ≤1 mg/L, treatment failure was only noted in three (20%). Thus, our results are compatible with the observations made in this small case study [10].

Other studies investigating effect of mecillinam in the treatment of (non-ESBL) UTI have reported lower overall treatment failure rates than ours [32,33], while others have reported comparable results [34,35]. Several factors may have contributed to an overall

Table 4. Independent risk factors of treatment failure in the mecillinam and the non-mecillinam treatment group.

Treatment group and variable	Level	Adjusted OR	95% CI	p-value
Mecillinam group				
- ESBL-producing strain	Yes/no	3.2	1.3–7.8	0.009
- Mecillinam MIC[a]	Doubling of MIC	2.0	1.4–3.0	<0.001
Non-mecillinam group				
- Strain resistant to initial treatment	Yes/no	29.5	12–71	<0.001

[a]For each doubling concentration starting at 1 mg/L which is the lowest level reported by the VITEK-2 (BioMerieux) system.
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high rate of treatment failure in the present study. Firstly, the inclusion criteria did not exclude complicated UTIs. Secondly, only bacteriologically verified UTIs were included. This criterion selects towards complicated UTI as the diagnosis of sporadic CA-UTI in women is often not supported by urine culture in Norway. Thirdly, a large proportion of the infections were caused by ESBL-producing strains with multiple drug resistance. Fourthly, patients with new UTIs occurring within two weeks from the index UTI and receiving a new prescription would have been classified as treatment failures under this study protocol. Given that 72% of treatment failures occurred within seven days this effect is probably small. Finally, the mean age of the study population was relatively high compared to other studies probably due to indications for culturing as mentioned above [36].

Our study was observational and associations between variables and treatment failure are therefore susceptible to bias. Only 37% of invited patients accepted the invitation to participate in the study and 40% of these patients did not receive empirical treatment. We have limited information about non-participants except for age, but assume this is a non-differential bias since both treatment groups probably are affected the same way. Another bias that may affect patients in different treatment groups differently is side effects resulting in new prescriptions that will be recorded as treatment failures. Furthermore, some patients may have been contacted by their doctor's practice staff when susceptibility testing identified bacterial resistance against the initial antimicrobial agent. This may have resulted in additional prescription indicating initial treatment failure even if the patient had clinical improvement. However, the significant association between clinical outcome recorded during interviews and data from the prescription database strongly indicates that these effects have been limited and that change of treatment in most cases was guided by patient symptoms. This underlines the reliability of a repeated prescription within 14 days as a valid surrogate marker for treatment failure. The patients were not randomized between treatment schemes. However, it is unlikely that this has affected the overall outcome since ESBL status was not known prior to treatment and patients with prior ESBL-positive infection were not included. Furthermore, the choice of treatment (type and duration) did not seem to be affected by ESBL status (data not shown). Finally, TEM-1 has a hydrolytic activity against mecillinam [14]. This enzyme may be present in ampicillin resistant strains including ESBL-producing strains. The OR for mecillinam treatment failure in non-ESBL producing ampicillin resistant versus non-ampicillin resistant strains was 2.0 (95% CI: 0.68–5.7, $p = 0.21$). Characterization of mechanisms of ampicillin resistance or identification of possible narrow spectrum bla_{TEM} or bla_{SHV} genes in ESBL-producing strains was not performed and could not be accounted for in the analyses performed. Thus this is a potential source of bias in the study.

Mecillinam has been proposed as an anti-ESBL agent [12]. The present study indicates that mecillinam with the current dosing (200 mg TID of pivmecillinam) has limited efficacy against infections caused by ESBL-producing *E. coli*. Although this is an observational study, we suggest that per oral mecillinam (i.e. pivmecillinam) should only be prescribed in uncomplicated UTIs caused by ESBL-producing *E. coli* if no other per oral options are available. We also suggest that higher doses of pivmecillinam than usually prescribed in Norway (200 mg TID) should be used because of the observed MIC-dependant efficacy. This is in particular relevant for patient at high risk of UTI caused by an ESBL-producing strains [16]. Significantly higher doses are manageable since pivmecillinam has a low toxicity. Our data also suggest that the mecillinam MIC break points for ESBL-producing *E. coli* should be reconsidered because of its reduced clinical efficacy and bactericidal effect against these strains.

Importantly the study results do not affect mecillinam's status as a first line drug in the empirical treatment of CA-UTI. The overall treatment failure rate was lower in patients receiving mecillinam (22%) than for patients in the non-mecillinam treatment (36%). This difference between the mecillinam and non-mecillinam group was valid also with different ESBL status (44% vs. 63% treatment failure in the ESBL group and 14% vs. 29% treatment failure in the non-ESBL group for patients in the mecillinam group and non-mecillinam group, respectively). This is probably because of the high prevalence of resistance to the other first-line per oral antibacterial drugs most commonly used against CA-UTI (Table 1).

In conclusion, we observed a high rate of mecillinam treatment failure in CA-UTI caused by ESBL-producing *E. coli* even for *in vitro* sensitive strains. The treatment failure of mecillinam was associated with ESBL-production per se as well as the increased MIC for mecillinam in ESBL-producers. Mecillinam is ecologically favourable and has a well documented effect in CA-UTI caused by non-ESBL producing *E. coli*. Further studies addressing the use of pivmecillinam against ESBL-producing *E. coli* with emphasis on optimal dosing and effect of combination therapy with β-lactamase inhibitors seem warranted.

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Author Contributions

Conceived and designed the experiments: ASO ASU PJ. Performed the experiments: ASO ASU PJ. Analyzed the data: ASO ASU SJ KL PAJ. Contributed reagents/materials/analysis tools: ASO ASU PJ. Wrote the paper: ASO ASU SJ KL PAJ.

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